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Function in Breast Cancer

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## **Introduction**

In recent years, ubiquitin-mediated protein degradation has been identified as a major regulator of the expression of numerous proteins. These proteolysis pathways are often responsible for the degradation of specific protein targets, as determined by specific mediators. For example, the E3-ligase MDM2 responsible for the degradation of p53 (2) while other cell cycle regulators, such as p27<sup>KIP1</sup>, are degraded by other proteins (5). It is becoming more apparent that ubiquitin-mediated proteolysis is responsible for the regulation of the levels of a wide variety of cellular proteins, and dysregulation of this process can be detrimental to cellular homeostasis, and eventually lead to the development of cancer. The subsequent loss of protein function can lead to cellular imbalances and tumor development. Although some loss of protein function can be attributed to genetic deletion or mutation, loss of some proteins (i.e. p27<sup>KIP1</sup>) can only be accounted for by accelerated ubiquitin-mediated proteolysis (5). Thus, this process is becoming a very important force driving cancer research. Identification of novel proteins involved in the ubiquitin pathway which are overexpressed in cancers may lead to a better understanding of disease progression and potentially give rise to new targets for drug therapy. RNF11 may be involved in the ubiquitin pathway by virtue of its ring finger and PY domains which can bind SCF- and HECT-type E3 ligases, respectively. RNF11 is highly overexpressed in breast cancers (6), however the mechanisms behind this overexpression and the resultant consequences remain unclear.

## **Body**

The primary amino acid sequence of RNF11 has been published (4; Fig.1). Bioinformatical analyses of this sequence revealed multiple important regulatory regions, notably a protein kinase B (PKB/AKT) phosphorylation site at threonine 135 (T135) which is located within a 14-3-3 binding motif (Fig. 1). This type of sequence has been reported for other proteins (i.e. BAD; 1) and seemed a logical place to start our investigation into the potential roles of AKT and 14-3-3 in the regulation of RNF11 function.

RNF11 is elevated in 90% of all invasive mammary ductal carcinomas that we evaluated (6). This strongly suggested a role for RNF11 in breast cancer. Since cancer is invariably a disease involving misregulation of the cell cycle in some way, we evaluated RNF11 gene expression across the cell cycle (Fig. 2). MCF-7 cells were arrested in G0 by serum and estrogen withdrawal for 48 hours. Cell cycle entry was induced by replenishing the serum and adding estradiol. Total RNA was extracted at various time points following serum addition and 10 µg of RNA was probed with a <sup>32</sup>P-labeled RNF11 cDNA probe (Fig. 2A). RNF11 mRNA levels are very low in G0 and rise as the cells progress through the cell cycle reaching a maximum at the G1/S transition and remaining elevated through to G2/M. This cyclical mRNA expression may be due to transcriptional activation by ETS1, as RNF11 has 2 ETS1 binding sites in its promoter (not shown). This pattern of expression combined with the importance of ETS1 in the cell cycle may hint at a potential function for RNF11 in cell cycle progression. To measure RNF11 cell cycle protein expression, MCF-7 cells were arrested as above except cells were transfected with FLAG-RNF11 expression vectors prior to arrest (Fig. 2B). FLAG-RNF11 protein followed a similar pattern of expression as RNF11 mRNA. The fact that FLAG-RNF11 was under the control of an exogenous promoter, thereby removing any mRNA regulation effects observed in Fig. 2A, suggests potential post-transcriptional mechanisms regulating RNF11 protein expression.

Our original idea was to study the role of AKT in regulating RNF11. RNF11 is overexpressed in breast cancers and AKT activation in breast cancers is associated with poor patient prognosis (3). In addition, activated AKT renders cells unresponsive to TGF-β, a condition that is common to many breast cancers. This fact combined with the potential role for RNF11 in the TGF-β signaling pathway makes this AKT/RNF11 connection plausible. To determine whether RNF11 is phosphorylated by AKT 2 relatively straightforward experiments were conducted (Fig. 3). WM239 cells, which possess elevated AKT activity, were transfected with GST-RNF11 vectors and lysates were subjected to GST-pulldowns. GST-RNF11 bound proteins were then probed with an antibody that recognizes phosphorylated AKT

substrates (Fig. 3A). These experiments revealed a 45 kDa band that corresponded to GST-RNF11. The levels of phospho-AKT substrate detected were reduced in cells where AKT activity was reduced by treatment with the PI3-kinase inhibitor LY294002. To further corroborate these findings, HIS-tagged RNF11 proteins purified from bacteria were phosphorylated *in vitro* by incubation with recombinant AKT and  $^{32}\text{P}$ - $\gamma$ ATP (Fig. 3B, lane 3). A 30 kDa band, which was subsequently identified as RNF11 by MS-MS mass spectrometry, was competed away by excess cold ATP (lane 1) and by incubation at 4°C (lanes 1 & 2). To show specificity, similar reactions were conducted with BCA2, a ring-finger protein which also contains a consensus AKT-binding site. This revealed 2 bands (lanes 4 & 5) which correspond to BCA2 on Western blots (not shown). GSK3- $\beta$  was also included as a positive AKT control (lane 6). Together, these results suggest that RNF11 is indeed a target for phosphorylation by AKT. To determine whether AKT phosphorylates RNF11 *in vivo*, 2-dimensional phosphopeptide maps for RNF11 were generated (Fig. 4). Wild-type (WT) RNF11 was transfected into WM239 cells, which have elevated AKT activity, and incubated with  $^{32}\text{P}$ . RNF11 was immunoprecipitated, run on SDS-PAGE gels, the RNF11 band was excised and excised proteins were trypsinized. RNF11 fragments were separated in 2 dimensions and resulted in the appearance of 7 distinct phosphorylated fragments (Fig. 4A). Inhibition of AKT with LY294002 resulted in a dramatic reduction in the levels of spot number 4, suggesting that this fragment was phosphorylated by AKT (Fig. 4B). To determine whether this was our suspected AKT site (T135), we generated mutant T135E proteins, which would prevent phosphorylation by AKT at this residue (Fig. 4C). This mutation resulted in the loss of spot 4 along with the unexpected loss of spots 1-3 and 5.

To establish whether RNF11 bound to 14-3-3, normal dermal epithelial cells (WM35) and malignant WM239 cells were transfected with expression vectors for GST-RNF11 (Fig. 5A). GST-pulldowns from cell lysates showed that the higher AKT activity in the WM239 cells compared to WM35 (Fig. 5B) resulted in a greater amount of 14-3-3 bound to RNF11. This was evident even before factoring in the approximately 8-fold less RNF11 present in WM239 cells. RNF11/14-3-3 interactions

were reduced when WM239 cells were treated with LY294002. To further corroborate these findings, MCF-7 cells were co-transfected with FLAG-RNF11 and equal amounts of either constitutively active (CA) or dominant negative (DN) AKT (Fig. 5C). Immunoprecipitation of FLAG-RNF11 revealed that more 14-3-3 was bound in the presence of CA AKT than in the presence of DN AKT, further suggesting that phosphorylation of RNF11 by AKT mediates binding to 14-3-3. Since the T135E mutant RNF11 exhibited diminished phosphorylation, the effects of this mutation on 14-3-3 interactions was examined (Fig. 5E). WT and T135E FLAG-RNF11 vectors were transfected into WM239 cells. As expected, strong WT RNF11/14-3-3 interactions were evident, and this binding was reduced upon the addition of LY294002. This RNF11/14-3-3 binding was inhibited by the T135E mutation, showing the importance of phosphorylation at this site in RNF11 binding to 14-3-3.

Since 14-3-3 can mediate the shuttling of proteins in and out of the nucleus, the effects of AKT and the subsequent increased 14-3-3 interactions, on RNF11 subcellular localization were measured (Fig 6A). In MCF-7 cells, RNF11 is predominantly cytoplasmic (Fig. 6A). The addition of CA AKT reduced RNF11 levels (not shown) and resulted in an almost entirely nuclear localization. T135E mutant RNF11 was also nuclear (Fig. 6A) but was expressed to similar levels in the presence or absence of CA AKT (not shown). Thus, there is definitely an effect of AKT on RNF11 localization. It could be that phosphorylation by AKT could result in an accelerated import into or a decreased export from the nucleus. However, this seems unlikely, since T135E RNF11 was also almost exclusively nuclear. The other possibility could be that AKT phosphorylation causes degradation of the cytoplasmic portion of RNF11. This may be the case since the elevated AKT activity in WM239 cells reduced RNF11 expression compared to that in control WM35 cells (Fig.5A). To determine whether this was a result of accelerated proteolysis of RNF11, WM239 cells were transfected with WT RNF11 in the presence and absence of the proteasome inhibitor MG132 (Fig. 6B). MG132 increased the levels of RNF11 protein to levels that were similar to those of the T135E RNF11, which is not phosphorylated by AKT. This suggests that phosphorylation of RNF11 by AKT at T135 may lead to its degradation.

### **Key Research Accomplishments**

1. Generation of a phosphomap for RNF11 showing multiple phosphorylation sites.
2. Establishment of AKT as the kinase responsible for phosphorylating RNF11 at T135.
3. Showing that AKT phosphorylation of RNF11 mediates its binding to 14-3-3.
4. Illustrating a role of the AKT kinase pathway in regulating RNF11 subcellular localization and/or degradation.
5. Publication of 2 research papers/reviews, resubmission of a previously submitted paper and the writing of a manuscript essentially completed for submission.

### **Reportable Outcomes**

1. **Connor, M.K.** and A. Seth. A Central Role for the Ring Finger Protein RNF11 in Ubiquitin-mediated Proteolysis via Interactions With E2s and E3s. *Oncogene* **23**, 2089-2095, 2004. See Appendix 1.
2. Freyssenet, D., I. Irrcher, **M. K. Connor**, M. Di Carlo and D. A. Hood. Calcium-regulated changes in mitochondrial phenotype in skeletal muscle cells. *Am. J. Physiol. (Cell Physiol.)* **286**, C1053-C1061, 2004. See appendix 1.
3. Sandhu, C., **M.K. Connor**, T. Kislinger, J.M. Slingerland and A. Emili. Global expression profiling of proliferating MCF-7 breast cancer cells. Submitted to Proteomics, May, 2004.
4. **Connor, M.K.** and A. Seth. AKT-mediated regulation of ring finger protein 11 (RNF11). *Biochem. Cell Biol.* In press, 2004. Abstract submitted for the 47<sup>th</sup> annual Canadian Society of Biochemistry and Molecular & Cellular Biology meeting (May 27-30, 2004). See appendix 1.
5. Applied for Molecular Cell Physiology Assistant Professor position at York University, Department of Kinesiology and Health Sciences, Dec. 2003. Currently in negotiations with the Dean of Science to start sometime in 2004.

### **Conclusions**

This research project was designed to characterize a novel protein that is overexpressed in breast cancer. RNF11 is a ring-finger protein that is phosphorylated by AKT and whose function is consequently regulated through this pathway. Via its interaction with Smurf2 (6) and Smad4 (unpublished) RNF11 likely plays an important role in TGF- $\beta$  signaling. To date, Aim 1 of my statement of work has been completed and significant progress on Aims 2 and 3 has been made. Current experiments are evaluating the effects of RNF11 on multiple aspects of TGF- $\beta$  signaling and how AKT alters these effects. Elucidation of the roles of RNF11 in TGF- $\beta$  signaling may allow for the identification of potential novel pharmaceutical therapeutic targets.

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## **Figure Legends**

**Figure 1. The RNF11 sequence contains multiple regulatory elements.**

**Figure 2. RNF11 is regulated across the cell cycle.** MCF-7 cells were synchronized in G0 by serum withdrawal for 48 hrs. Cell cycle entry was achieved by the addition of serum. Total RNA was isolated from cell at various times across the cell cycle and 10 µg of RNA was run on a Northern blot. Blots were probed with a <sup>32</sup>P-labelled RNF11 cDNA probe. To determine whether RNF11 protein levels varied across the cell cycle, MCF-7 cells were transfected with FLAG-RNF11 expressing vectors and cells were arrested as above and 25 µg of whole cell lysates were immunoblotted for FLAG-RNF11.

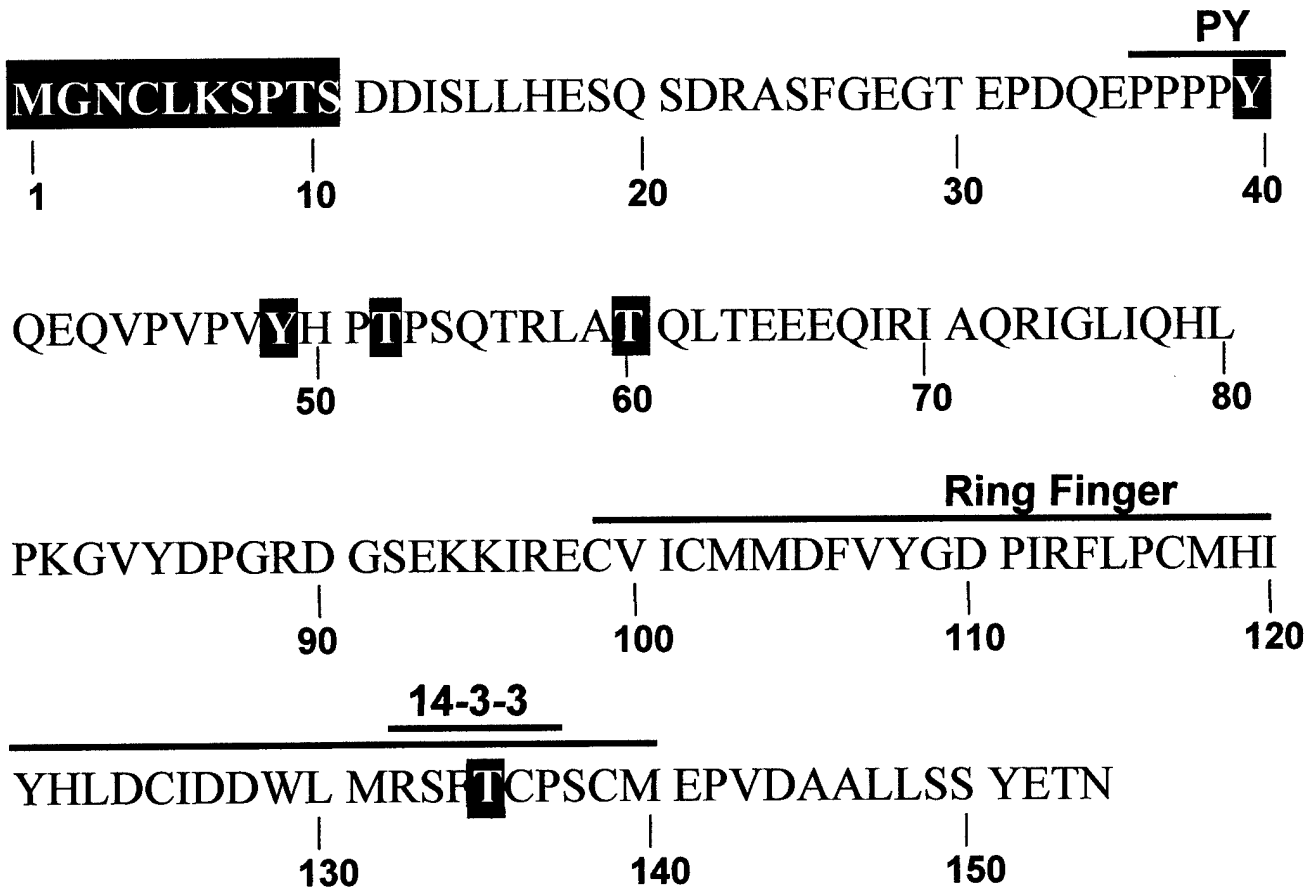
**Figure 3. RNF11 is phosphorylated by AKT.** A) WM239 cells were transfected with GST-RNF11. Lysates were subjected to GST-pulldowns and RNF11 bound proteins were probed with a phospho-AKT substrate antibody. The amount of phospho RNF11 recognized by this antibody is greater in WM239 cells (PTEN deletion) than in the parental WM35 cells. Treatment of WM239 cells with LY294002 reduced this antibody interaction. B) His-tagged RNF11 was amplified and isolated from bacteria using Nickel columns. Purified His-RNF11 was incubated with recombinant Akt and <sup>32</sup>P-γATP (lane 3). As negative controls, reactions were run in the presence of excess cold ATP (lane 1) or at 4°C (lane 2). GSK3-β was run as a positive control (lane 6) and BCA2, which possesses a consensus Akt site, was included (lane 5) to show specificity of phosphorylation.

**Figure 4. RNF11 is phosphorylated at multiple sites.** A) FLAG-RNF11 was labeled with <sup>32</sup>P in WM239 cells, which possess elevated AKT activity, and subjected to digestion with trypsin. RNF11 fragments were then subjected to 2-dimensional electrophoresis. B) RNF11 was prepared as in A) except cells were treated with LY294002 to inhibit AKT activity. C) Same as A) except T135E mutant RNF11 was used instead of wild-type (WT).

**Figure 5. RNF11 binding to 14-3-3 is regulated by AKT.** A) Normal WM35 and malignant WM239 (high AKT activity) were transfected with GST-RNF11 in the presence or absence of LY294002 and subjected to GST-pulldowns to isolate RNF11-bound proteins. Pulldowns were immunoblotted for 14-3-3 and GST. B) Whole cell extracts from A) were probed for activated AKT. C) Whole cell extracts (W.C.E.) from MCF-7 cells transfected with FLAG-RNF11 and either dominant negative (DN) or constitutively active (CA) AKT. (D) RNF11 was immunoprecipitated from lysates in (C) and probed for 14-3-3 and FLAG. (E) Lysates were prepared as in A) using wild-type (WT) or T135E mutant RNF11 expression vectors.

**Figure 6. Phosphorylation by AKT promotes degradation of cytoplasmic RNF11.** (A) MCF-7 cells were transfected with either wild-type (WT) or T135E mutant FLAG-RNF11 in the presence or absence of the proteasome inhibitor MG132. (B) MCF-7 cells were transfected as in (A) either with or without transfected constitutively active Akt (CA). Cells were separated into nuclear and cytoplasmic components by digitonin permeabilization and probed for FLAG-RNF11.

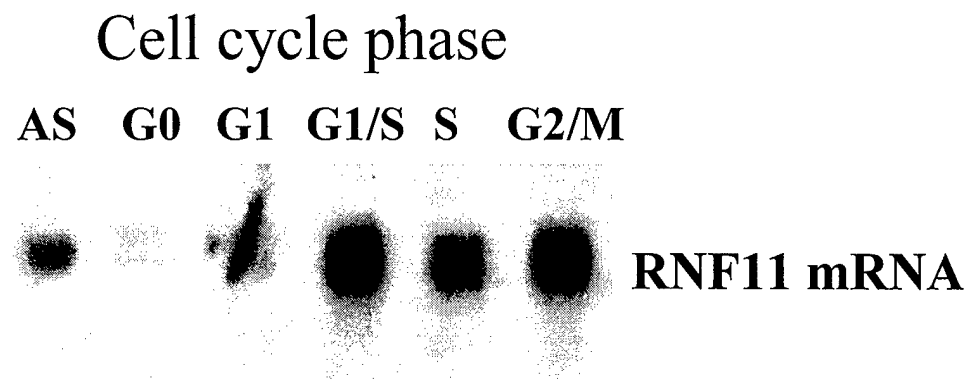
## RNF11 Sequence



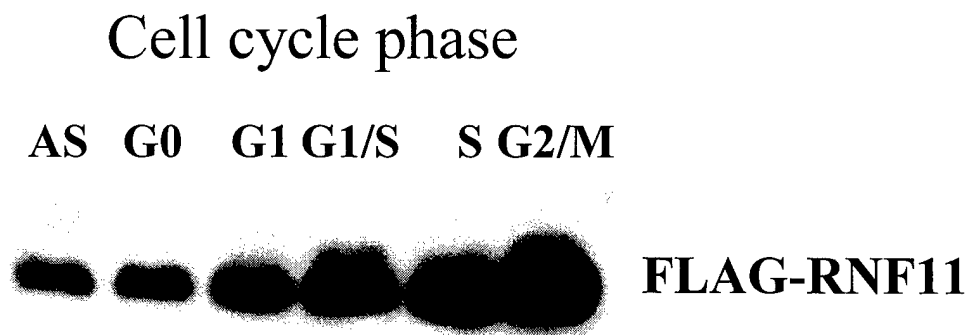
- Murystoylation Site
- EGFR Kinase Site
- GSK3- $\beta$  Site
- AKT Site

**Figure 1**

**A**

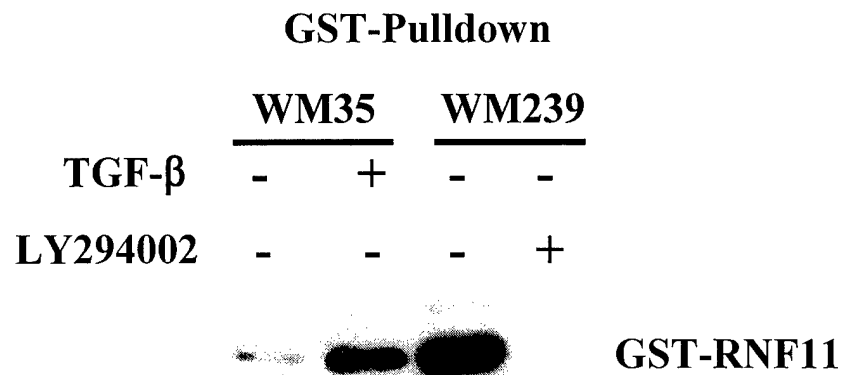


**B**



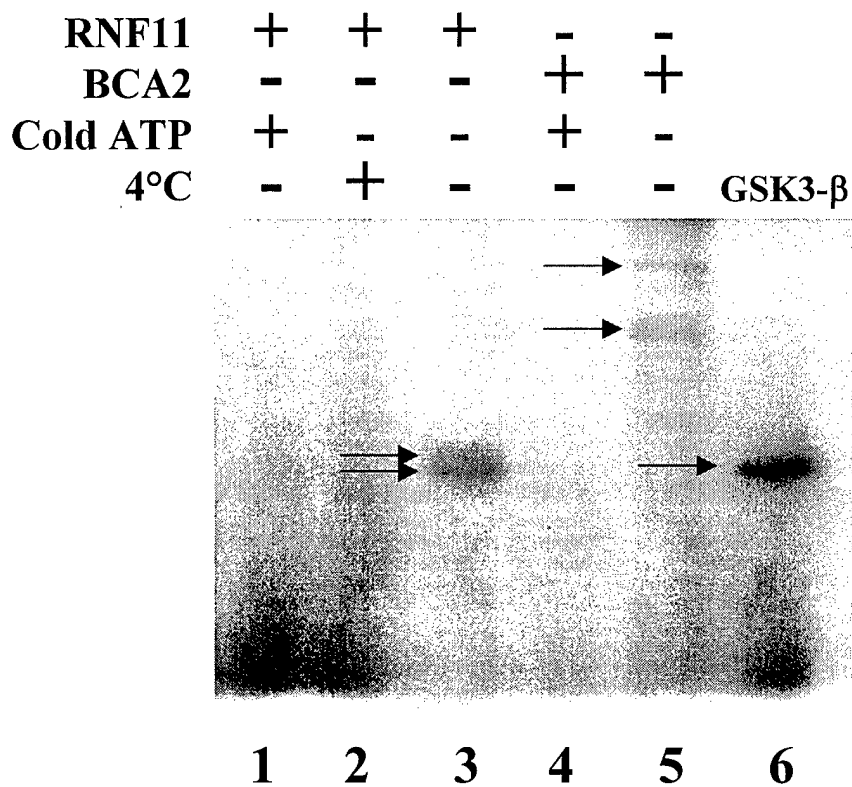
**Figure 2**

**A**



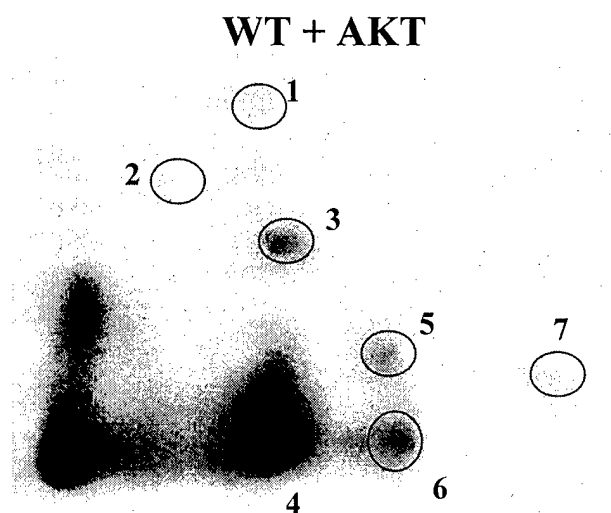
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**B**

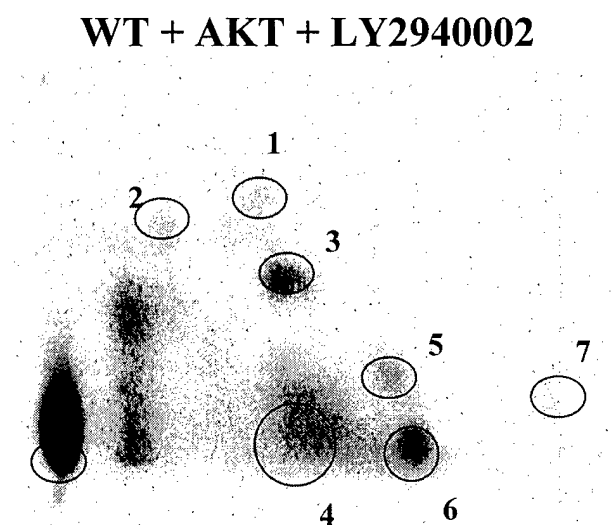


**Figure 3**

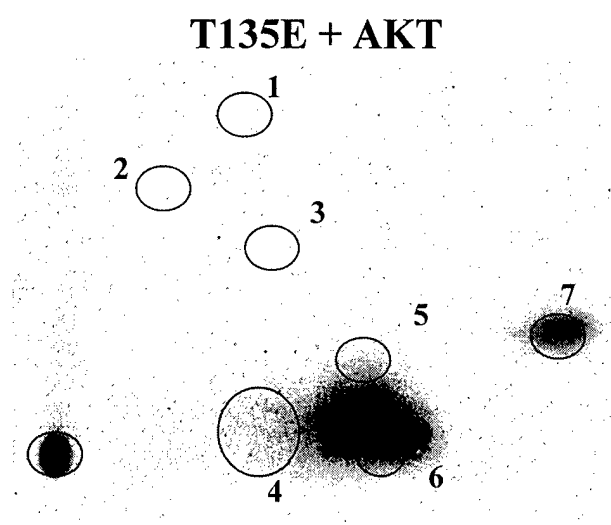
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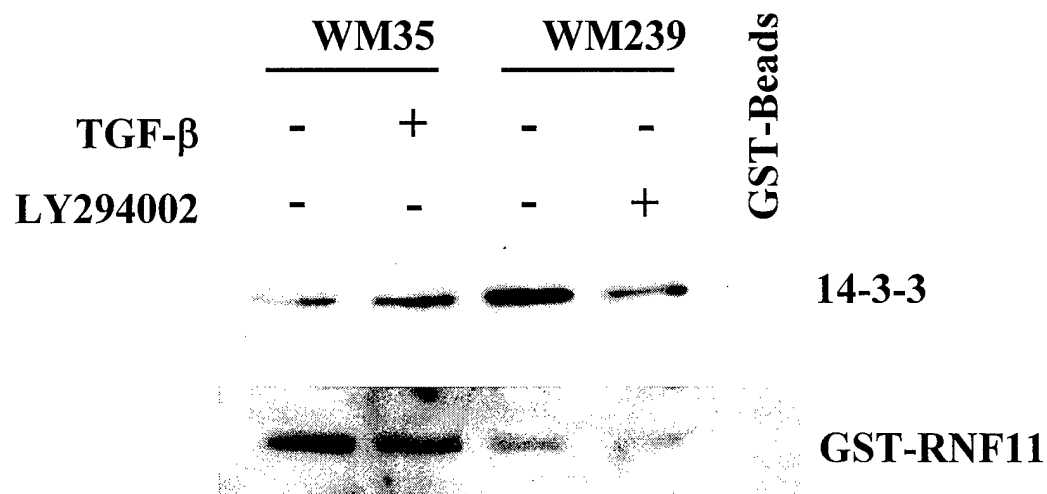
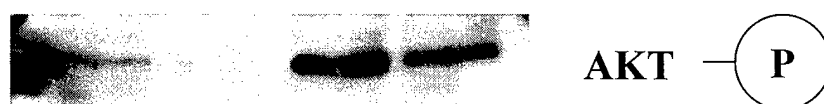
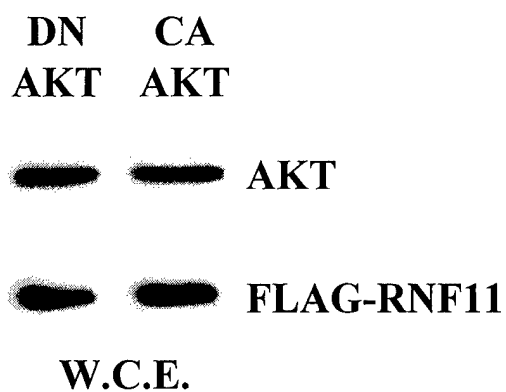
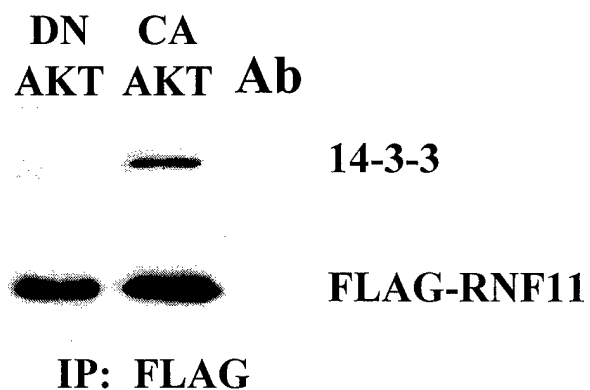
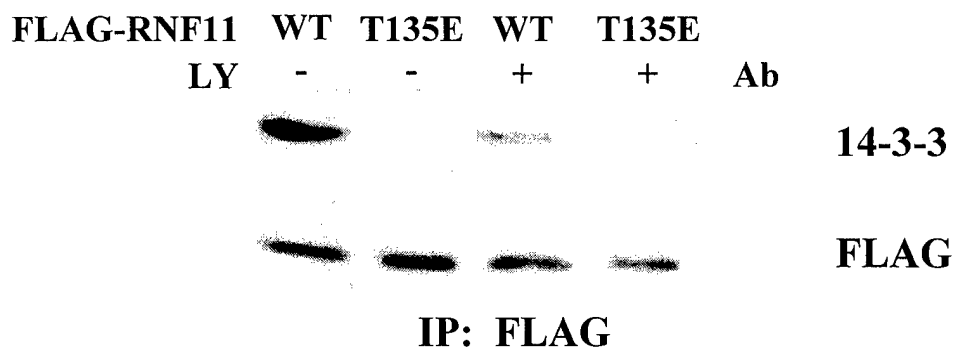
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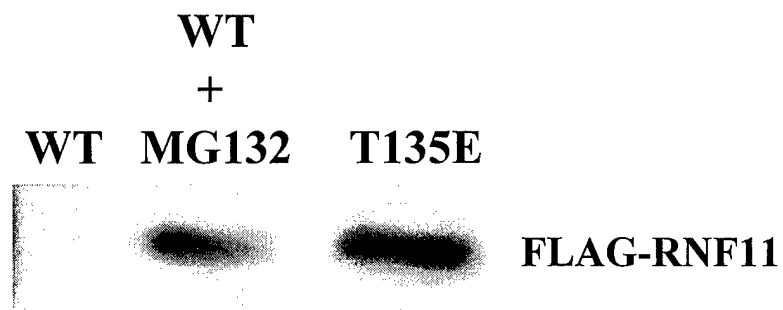
**C**



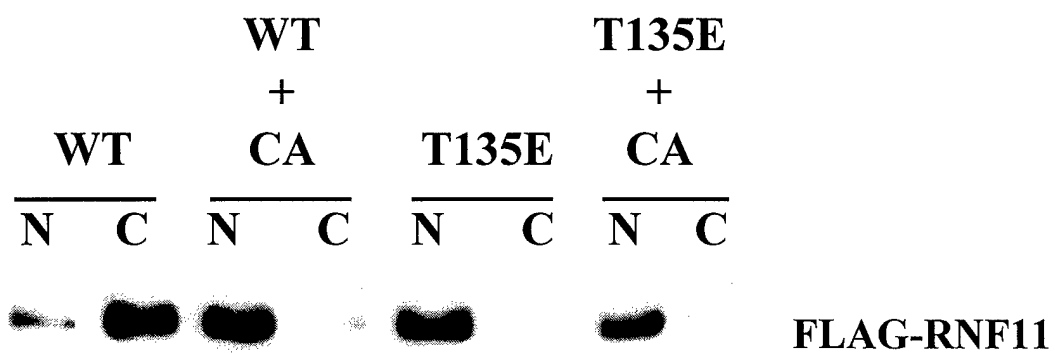
**Figure 4**

**A****B****C****D****E****Figure 5**

**A**



**B**



**Figure 6**

## **APPENDIX1**

## A central role for the ring finger protein RNF11 in ubiquitin-mediated proteolysis via interactions with E2s and E3s

Michael K Connor<sup>1</sup> and Arun Seth<sup>\*1,2</sup>

<sup>1</sup>Molecular and Cellular Biology Research, Laboratory of Molecular Pathology, Rm E-423b, Sunnybrook & Women's College Health Sciences Centre, Toronto, Ontario, Canada; <sup>2</sup>Department of Laboratory Medicine and Pathobiology, the CIHR Group in Matrix Dynamics, University of Toronto, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5

The identification of novel tumor-associated genes represents an important area of cancer research. To that end, we have discovered a number of genes whose expression is altered in breast tumors. One of these genes has been identified as the ring finger protein 11 (RNF11) and its expression is elevated in breast and prostate cancer. The RNF11 gene encodes a 154 amino-acid protein that contains a ring finger and a PY motif. RNF11 is capable of binding numerous proteins, which encompass a wide variety of cellular pathways and mechanisms. This gives RNF11 a corresponding breadth of functions, including involvement in TGF- $\beta$  and epidermal growth factor receptor (EGFR) signaling. In addition, RNF11 has the potential to mediate the ubiquitination and subsequent proteolysis of many cellular proteins. Thus, it may represent an important target of novel cancer therapies. *Oncogene* (2004) 23, 2089–2095. doi:10.1038/sj.onc.1207380

**Keywords:** RNF11; ubiquitination; Smurf2; TGF- $\beta$ ; breast cancer; signaling; EGFR

### Ubiquitin-mediated degradation and cancer

In recent years, ubiquitin-mediated protein degradation has been identified as a major regulator of the stability or abundance of numerous proteins. Ubiquitin-mediated degradation is a complex process that is comprised of numerous well-defined steps (Joazeiro and Weissman, 2000; Weissman, 2001; Nalepa and Harper, 2003). Initially, ubiquitin is activated by an ubiquitin-activating enzyme (E1) and is subsequently transferred to an ubiquitin-conjugating enzyme (E2). Ubiquitin is then transferred to its target protein either by an E2 or by an ubiquitin ligase (E3). This process cycles repeatedly and the resultant polyubiquitin chains target the protein to the proteasome for degradation. E3 ligases are classified as HECT-, RING- or U box-type (Joazeiro and Weissman, 2000; Weissman, 2001; Hatakeyama and Nakayama, 2003). HECT-type E3 ligases (Smurf2, Nedd4, AIP4 and NEDL2) contain WW

domains, which bind to PY motifs in other protein partners (i.e. Smad2, Smad3, p73). HECT-type ligases are themselves ubiquitinated by E2s, and subsequently transfer this ubiquitin to the target protein. RING-type E3 ligases are subdivided into those that are capable of causing ubiquitylation on their own (Mdm2, Cbl) or those that act as part of a multisubunit E3 complex (ROC1, APC11). The relatively novel U-box proteins (Aravind and Koonin, 2000; Hatakeyama *et al.*, 2001) function similar to RING-type E3s, but mediate some atypical polyubiquitin linkages (Hatakeyama and Nakayama, 2003). The specificity of these E3 ligases has been discussed elsewhere (Fang *et al.*, 2000) and the importance of these proteins in the regulation of proper cellular function is well established.

It is becoming more apparent that ubiquitin-mediated proteolysis is responsible for the regulation of the levels of a wide variety of cellular proteins. The deregulation of ubiquitin-mediated proteolysis can be detrimental to cellular homeostasis. The resultant loss of protein function can lead to cellular imbalances and tumor development (Sakamoto, 2002). Thus, this process is becoming a very important force driving cancer research (Gillesen *et al.*, 2002). Although some losses of protein function in tumors can be attributed to genetic deletion or mutation, loss of some proteins (i.e. p27<sup>KIP1</sup>) can only be accounted for by accelerated ubiquitin-mediated proteolysis (Slingerland and Pagano, 2000). In fact, there is an association between the expression of components of the ubiquitin proteolysis pathway and cancer progression. Increased levels of E3 ligases have been observed in numerous different tumor types. The F-box protein Skp2, which is responsible for the degradation of the cdk inhibitor p27<sup>KIP1</sup>, is important in breast and other cancers (Gstaiger *et al.*, 2001; Ben-Izhak *et al.*, 2003; Shigemasa *et al.*, 2003). It is well established that loss of p27<sup>KIP1</sup> is associated with poor survival of cancer patients (Catavelos *et al.*, 1997). Therefore, the overexpression of Skp2 is a deleterious adaptation/alteration in cancer cells. Other examples of the deregulation of E3 activity in cancers have also been reported. For example, the overexpression of the HECT-type ligase Smurf2, which degrades multiple components of the TGF- $\beta$  signaling pathway, has been reported in esophageal squamous cell carcinoma and is associated with poor patient prognosis (Fukuchi *et al.*,

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2002). In addition, Mdm2, the E3 responsible for the degradation of p53, is overexpressed in 5–10% of all cancers (Michael and Oren, 2002; Fang *et al.*, 2003). Thus, it is becoming increasingly apparent that E3 ubiquitin ligases represent potential targets for novel cancer therapies (Momand *et al.*, 1998; Pray *et al.*, 2002).

### The TGF- $\beta$ signaling pathway

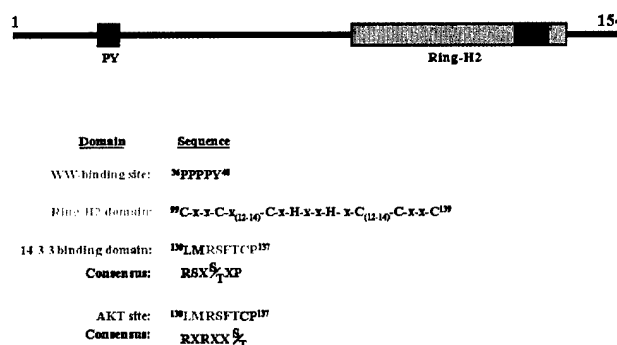
TGF- $\beta$  is secreted from cells into the extracellular matrix where it can bind to TGF- $\beta$  receptors on the plasma membrane of neighboring cells (Liu *et al.*, 2001; Medrano, 2003). TGF- $\beta$  is a member of the TGF- $\beta$  superfamily of proteins including TGF- $\beta$ , bone morphogenetic protein (BMP) and activin. The TGF- $\beta$  signaling pathway is complex and involves positive regulators (R-Smads and co-Smads) and negative regulators (I-Smads and Smurfs). Binding of TGF- $\beta$  to the TGF- $\beta$  receptors (T $\beta$ RI and T $\beta$ RII) causes a transphosphorylation and activation of the T $\beta$ RI by T $\beta$ RII. This leads to the phosphorylation of the receptor-activated Smads (R-Smads) including Smads 2 and 3, which subsequently bind to Smad4. This complex is transported to the nucleus where it induces transcription of TGF- $\beta$  responsive genes (Mehra and Wrana, 2002). One of the mechanisms of regulating TGF- $\beta$  signaling involves Smad7 and Smurf2 (Kavsak *et al.*, 2001; Nakao *et al.*, 1997). Smad7 binds to Smurf2 (Kavsak *et al.*, 2001) and this complex is responsible for the degradation of T $\beta$ RI, T $\beta$ RII and the R-Smads, thereby silencing TGF- $\beta$  signaling (Hayashi *et al.*, 1997; Kavsak *et al.*, 2001). One important consequence of the activation of the TGF- $\beta$  pathway is a G1 cell cycle arrest. In many cancers, tumor cells lose the responsiveness to TGF- $\beta$ , leading to the idea that the disruption of this pathway may be an important factor in the development of cancer (Massague *et al.*, 2000; Miyazono *et al.*, 2003). In support of this, many pancreatic and colon cancers demonstrate Smad4 deletions (Miyaki *et al.*, 1999). In addition, deletions of the T $\beta$ RII have been reported in colon cancers (Kretzschmar, 2000). However, this only represents a small fraction of the cancers that are insensitive to TGF- $\beta$ . Thus, there must be other factors that contribute to TGF- $\beta$  in cancer. These likely include accelerated degradation of the Smads or interference of Smad interactions, both of which would lead to a blunted response to TGF- $\beta$  (Xu and Attisano, 2000). Increased expression of Smad7 has been reported in pancreatic carcinomas and inflammatory bowel disease (Monteleone *et al.*, 2001). Thus, the inhibitory activity of Smad7 and Smurf2 appear to be important in disease states. There may be other as yet unidentified proteins that contribute to the deregulation of the TGF- $\beta$  signaling pathway and the accompanying TGF- $\beta$  insensitivity evident in cancer cells.

### Ring finger protein 11 (RNF11) in the ubiquitin pathway

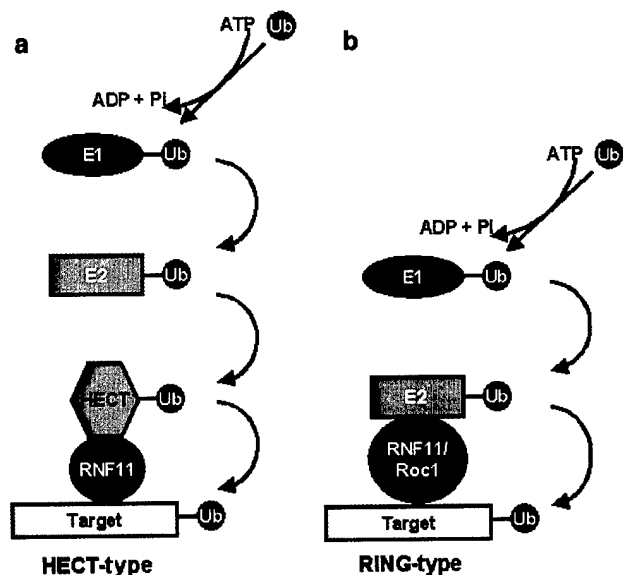
Identification of novel proteins involved in the ubiquitin pathway that are overexpressed in cancers may lead to a

better understanding of disease progression and potentially give rise to new targets for drug therapy. To that end, we have identified multiple genes from a cDNA library enriched for tumor mRNAs that are elevated in breast cancer (Burger *et al.*, 1998). One of these clones was identified as RNF11, a 154 amino-acid protein that contains a ring finger in between amino acids 98–140 (Seki *et al.*, 1999; Figure 1). Other important regions are the N-terminal PY motif and a 14-3-3 binding motif located within the ring domain. We have shown that RNF11 is capable of binding numerous proteins, including those involved in the TGF- $\beta$  and ubiquitin pathways (Kitching *et al.*, 2003; Li and Seth, 2003; Subramaniam *et al.*, 2003). We have also reported that RNF11 is highly overexpressed in breast and pancreatic cancers, and moderately overexpressed in head and neck, colon and lung cancers (Subramaniam *et al.*, 2003). However, the mechanisms behind this overexpression and the resultant consequences remain unclear.

Most of our observations of RNF11 suggest a role in ubiquitin-mediated proteolysis via its interaction with HECT-type E3 ligases (Li and Seth, 2003; Subramaniam *et al.*, 2003). Interestingly, RNF11 is also capable of binding to Cull1, a core member of the SCF (Skp1/Cul1/F-box protein) complex (Subramaniam and Seth, unpublished observations). This gives RNF11 a potentially important role in protein degradation by HECT- and SCF-type E3 ligases. RNF11 may function in a manner similar to the ring finger protein ROC1 in SCF-mediated ubiquitination (Joazeiro and Weissman, 2000). However, whether it be in HECT- or SCF-mediated degradation pathways, RNF11 appears to be important for giving specificity to proteolytic mechanisms (Figure 2). We also observe binding of RNF11 to the E2s UbcH5 a, b and c (Subramaniam *et al.*, 2003). This is the first evidence of a protein binding to both E2 and E3 molecules. When bound to UbcH5, RNF11 facilitates the ubiquitination of Smurf2 and of itself (Subramaniam *et al.*, 2003). Thus, it appears that UbcH5 may be the E2 responsible for promoting the RNF11-mediated ubiquitination of Smurf2. The full ramifications of RNF11 interaction with both E2 and E3 proteins need further investigation. This review will focus mainly on the involvement of RNF11 in ubiquitin-



**Figure 1** Schematic drawing of the important sites and motifs in the RNF11 sequence



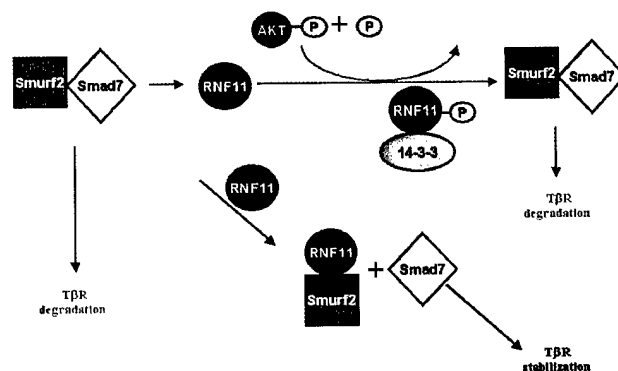
**Figure 2** Hypothetical models for how RNF11 is involved in HECT-type (a) and RING-type (b) ubiquitination of target proteins

mediated proteolysis and its various effects on cellular function, including TGF- $\beta$  and epidermal growth factor receptor (EGFR) signaling, and how RNF11 may impact cancer progression.

#### RNF11 in TGF- $\beta$ signaling

Through its PY motif, RNF11 can bind to Smurf2, thus making it a potential regulator of TGF- $\beta$  signaling. RNF11 may also compete with Smad7 binding to Smurf2, thereby disrupting the Smurf2/Smad7 complex (Hayashi *et al.*, 1997; Kavak *et al.*, 2001; Figure 3). This would prevent the negative effects of Smurf2 and Smad7 on TGF- $\beta$  signaling and act to restore TGF- $\beta$  sensitivity to cells that have lost TGF- $\beta$  responsiveness. In fact, when RNF11 is overexpressed in 293T cells there is an elevation in TGF- $\beta$  responsiveness, demonstrating the relevance of RNF11 in TGF- $\beta$  signaling (Subramaniam *et al.*, 2003).

Since breast cancers seem most susceptible to becoming TGF- $\beta$  resistant (Donovan and Slingerland, 2000; Kretschmar, 2000), the overexpression of RNF11 in these cancers may illustrate that the cell is trying to re-establish TGF- $\beta$  signaling. There are many cancers and cell lines that are resistant to TGF- $\beta$ , thus it appears that this strategy may not be entirely effective. Why is this so? The answer may lie partly within the RNF11 protein sequence. Near the carboxy terminus, there is a potential AKT phosphorylation site (T135). This site lies within a consensus 14-3-3 binding site (Figure 1). This motif is identical to one found in the HECT-type E3 ligase Nedd4 (Jolliffe *et al.*, 2000), and Nedd4 function has been shown to be regulated by AKT kinase activity (Boehmer *et al.*, 2003). It is known that phosphorylation of a protein within its 14-3-3 binding site can promote interaction with 14-3-3 (i.e. BAD phosphorylation by



**Figure 3** How RNF11 potentially interacts within the TGF- $\beta$  signaling pathway. Positive effects on TGF- $\beta$  signaling are shown in black, while negative effects on TGF- $\beta$  signaling are indicated in red

AKT; Datta *et al.*, 1999). An AKT-mediated sequestration of RNF11 away from Smurf2 by 14-3-3 may prevent RNF11 competition with Smad7 and result in the loss of sensitivity to TGF- $\beta$  (Figure 3). In malignant cell lines that are TGF- $\beta$  resistant, inhibiting AKT returns TGF- $\beta$  responsiveness to these cells (Liang *et al.*, 2002; Shin *et al.*, 2002; Viglietto *et al.*, 2002). This shows the importance of AKT in regulating TGF- $\beta$  sensitivity, and this may also be important in the regulation of RNF11 function. We have observed multiple RNF11 phosphopeptide fragments by 2-dimensional phosphopeptide analysis, which suggests that other phosphorylation events are also involved in mediating RNF11 function (Connor and Seth, unpublished).

By using yeast two-hybrid analyses, we have reported that RNF11 binds to numerous proteins involved in signaling, transcription and protein degradation (Li and Seth, 2003). RNF11 may have other effects on TGF- $\beta$ /BMP signaling through its interactions with the AMSH (associated molecule with the SH3 domain of STAM) protein (Tanaka *et al.*, 1999). AMSH has been shown to induce BMP-mediated transcription by binding to Smad6 (Itoh *et al.*, 2001). In a similar fashion, AMSH interacts with Smad7 in the TGF- $\beta$  pathway. TGF- $\beta$  stimulation enhances AMSH/Smad7 interactions, thereby preventing the degradation of the TGF $\beta$ R by the Smad7/Smurf2 complex. This is similar to the effect of overexpression of RNF11 on the TGF- $\beta$  pathway, where RNF11 enhances the activation of TGF- $\beta$  responsive promoters (Subramaniam *et al.*, 2003), possibly by stabilizing the T $\beta$ R complex by competing with Smad7 for binding to Smurf2, effectively disrupting and inactivating the Smad7/Smurf2 complex (Figure 3). It is interesting that while RNF11 and AMSH each act to prevent Smad7 from binding to Smurf2, RNF11 is also capable of causing the degradation of AMSH when complexed with Smurf2 (Li and Seth, 2003).

#### RNF11 in the EGFR pathway

The EGFR is a tyrosine kinase, which initiates cell growth and proliferation. Improper regulation of the

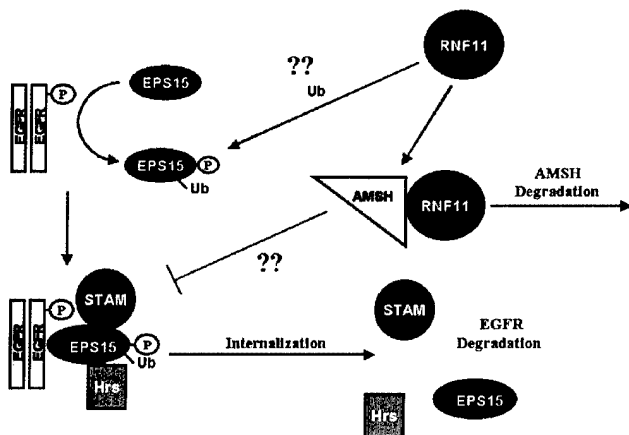
an important target in cases where increased EGFR signaling is responsible for accelerated cell proliferation and tumor progression.

### Other potential functions of RNF11

The interactions of RNF11 with both Smurf2 and AMSH have been recently described and some of the consequences of these interactions have been elucidated (Kitching *et al.*, 2003; Li and Seth, 2003; Subramaniam *et al.*, 2003). However, RNF11 binds with numerous other cellular proteins (Li and Seth, 2003). As of yet, the net results of these interactions remain unexplained. Some of the more conceptual functions of RNF11 are outlined below.

The protein-tyrosine kinase Syk plays an essential role in lymphocyte development and activation of immune cells (Mustelin and Tasken, 2003). Syk contains two Src homology 2 (SH2) domains in tandem and multiple autophosphorylation sites. Syk is activated upon binding of tandem SH2 domains to immunoreceptor tyrosine-based activating motif (ITAM) and Syk is critical for tyrosine phosphorylation of multiple proteins that regulate important pathways leading from the receptor, such as  $\text{Ca}^{(2+)}$  mobilization and mitogen-activated protein kinase (MAPK) cascades. Syk degradation is an ubiquitin-dependent process. In B cells, Syk binds to latent membrane protein 2A (LMP2A) via its SH2 domains in a phosphorylation-dependent manner (Mori *et al.*, 2003). LMP2A then acts as a scaffold bringing Syk into a complex with the Nedd4 family HECT-type E3 ligase AIP4 (Winberg *et al.*, 2000). The PY motif in LMP2A binds to the WW domain in AIP4, and recruits Syk for ubiquitination and its subsequent degradation by the 26S proteasome. However, Syk expression is not exclusive to B cells (Yanagi *et al.*, 2001). It is known that Syk functions as a tumor suppressor in that it can inhibit breast cancer cell growth and metastasis (Coopman *et al.*, 2000). We have demonstrated that RNF11 is capable of binding to AIP4 via its PY motif (Kitching *et al.*, 2003). Thus, it is possible that in epithelial cells RNF11 can mirror the function of LMP2A by bringing Syk and AIP4 together. It has been shown that in B cells Syk can activate PI-3 kinase-dependent activation of Akt. Akt activation has been implicated as an important factor in TGF- $\beta$ -resistant mammary epithelial cells. This may implicate Syk in TGF- $\beta$  resistance in breast cancer. If AIP4 and RNF11 couple to mediate Syk degradation, overexpression of RNF11 would play a role in promoting breast cancer progression (Subramaniam *et al.*, 2003). In addition to the potential role in Syk degradation, it is plausible that AIP4 can target other RNF11 binding partners (Kitching *et al.*, 2003; Li and Seth, 2003), leading to their ubiquitin-mediated degradation.

One of the most widely reported genetic mutations in cancer is the breast and ovarian cancer susceptibility gene 1 (BRCA1). BRCA1 is a transcriptional regulator that is involved in the DNA damage/repair pathway (Gilmore *et al.*, 2003; Moynahan, 2002; Rosen *et al.*, 2003). The protein ZBRK1 acts as a repressor of gene



**Figure 4** Potential roles for RNF11 in the EGFR signaling pathway. Positive effects on EGFR signaling are shown in black, while negative effects on EGFR signaling are indicated in red.

transcription, which is dependent on BRCA1 (Peng *et al.*, 2002; Zheng *et al.*, 2000). ZBRK1 is a 60 kDa KRAB-containing protein with eight central zinc fingers. RNF11 has been shown to interact with ZBRK1 (Li and Seth, 2003), suggesting a potential role for RNF11 in the BRCA1 pathway. RNF11 may serve to enhance the BRCA1 response by accentuating BRCA1/ZBRK1 interaction. It has been recently reported that ubiquitination of the HIV TAT protein enhances its transcriptional activity (Br s *et al.*, 2003). This may also be the case for ZBRK1. In contrast, RNF11 may have a negative effect on BRCA1 function by binding to ZBRK1, preventing its interaction with BRCA1. Alternatively, RNF11 may target ZBRK1 for destruction via ubiquitin-mediated proteolysis.

## Discussion

We originally identified RNF11 as a protein whose gene product was elevated in breast tumors (Kitching *et al.*, 2003). The RNF11 gene encodes a 154 amino-acid protein containing a ring finger and a PY motif. Our lab is focused on investigating the cellular function(s) of RNF11, and attempting to unravel the relevance of RNF11 overexpression in cancer. We have determined numerous RNF11 binding partners using yeast two-hybrid screening and sequence analysis, the nature of which suggests a number of complex and potentially important functions. These range from signaling to transcriptional regulation and suggest a central role for RNF11 in ubiquitin-mediated proteolysis (Kitching *et al.*, 2003; Li and Seth, 2003; Subramaniam *et al.*, 2003).

The two main regions within RNF11 are the PY motif (Figure 1), which interacts with WW domains of the Smurf2 and AIP4 HECT-type E3 ligases, and the ring finger domain, which is also an important region in mediating protein-protein interactions including with E2s (Figure 2; Subramaniam *et al.*, 2003). The PY motif is identical to that found in Smad7. Both RNF11 and Smad7 appear to compete for binding to the HECT-type E3 ligase Smurf2 through this PY region. We have yet to determine whether Smurf2 preferentially binds to RNF11 or Smad7, but it is clear that the stoichiometric balance between these three proteins is important. This competition for Smurf2 can have important effects on cell function. When complexed with Smad7, Smurf2 acts to degrade the TGF-  receptor. This would lead to a misregulation of the TGF-  signaling pathway, the likely result being insensitivity to TGF- , a characteristic of many breast cancer cells. If RNF11 can displace Smad7 from this complex, there would be a stabilization of the TGF-  receptor and a restoration of proper TGF-  signaling (Figure 3; Subramaniam *et al.*, 2003). Another interesting observation is that in the presence of overexpressed RNF11 and Smurf2, increasing the expression of Smad7 enhances the ubiquitination and degradation of both RNF11 and Smurf2 (Subramaniam and Seth, unpublished observations). However, it appears that the regulation of RNF11 function is more

complex. Within its C-terminal region, RNF11 contains a potential AKT site that is located within a 14-3-3 binding motif. This domain may be important in regulating RNF11 function. The 14-3-3 family of proteins are known to be 'molecular anvils', binding to target proteins and rigidly altering their conformation, and this binding depends upon the phosphorylation status of residues within the target protein binding site. RNF11 may therefore have separate and distinct functions depending upon the relationship between one or more AKT kinases and 14-3-3 proteins such that in one conformation RNF11 prevents TGF-  responsive growth arrest, and in another conformation it preserves TGF-  signaling by displacement of Smad7. Thus, the prevention of RNF11 phosphorylation by AKT within the 14-3-3 binding domain may be important for restoring TGF-  sensitivity in resistant cells.

We have identified numerous other binding partners for RNF11. One of these is the AMSH protein and the functional significance of this binding is potentially relevant to more than one signaling pathway. RNF11 can promote the ubiquitination and degradation of AMSH. AMSH can bind and sequester Smad6 and Smad7 from Smurf2 and thus act to maintain the integrity of the BMP and TGF-  signaling pathways, respectively. Accelerated degradation of AMSH could therefore result in the deregulation of these signaling pathways, which could have detrimental effects on cellular function including uncontrolled proliferation and malignant transformation. The stoichiometry of these proteins is likely critical in determining the net results on cellular function and may explain why so many breast cancers express elevated RNF11 protein levels (Subramaniam *et al.*, 2003).

AMSH also may play roles in EGFR signaling. AMSH binds to STAM and may prevent the formation of the complex necessary for EGFR degradation, which would prolong EGFR signaling and promote cellular proliferation (Figure 4). Interestingly, RNF11 may play a dual role in helping to maintain proper EGFR regulation. By inducing AMSH degradation, proper EGFR internalization may be restored. Also, EPS15 is a binding partner of RNF11, giving RNF11 a potential for involvement in EPS15 monoubiquitination. This is essential for EGFR internalization and degradation. Furthermore, RNF11 has two potential EGFR kinase sites. Thus, phosphorylation of RNF11 by the EGFR may be part of the negative feedback mechanism responsible for controlling EGFR activity.

ZBRK1 is another protein that binds to RNF11. ZBRK1 also binds to BRCA1 and acts to suppress gene transcription in response to DNA damage. The fact that RNF11 is overexpressed in breast cancer and BRCA1 is an important breast cancer gene may not be a coincidence. The relevance of the relationship between RNF11, ZBRK1 and BRCA1 remains to be established, but represents a potentially important avenue of RNF11 function.

We are just beginning to scratch the surface of the involvement of RNF11 in the ubiquitin proteolytic

pathway. Via its PY motif RNF11 can bind to at least three HECT-type E3 ubiquitin ligases (Kitching *et al.*, 2003; Li and Seth, 2003; Subramaniam *et al.*, 2003). This gives RNF11 the potential responsibility for mediating the degradation of a wide variety of cellular proteins. Additional observations give an indication of the complexity of the role of RNF11 in ubiquitin-mediated proteolysis. RNF11 binds to the E2 enzymes UbcH5 a, b and c, in addition to the HECT-type E3 ligases. This dual binding to E2 and E3 proteins suggests that RNF11 may be involved in bringing these proteins together for target ubiquitination, and this possibility remains an interesting focus of future investigations. Similar to ROC1, we see RNF11 binding to Cull1, a member of the SCF complex. Thus, it appears that the involvement of RNF11 in ubiquitin-mediated proteolysis is widespread. Determination of the precise conditions that regulate RNF11 interactions with various E2 and E3 proteins will provide valuable insight into the importance of RNF11 as a mediator of cellular protein turnover.

The significance of RNF11 overexpression in tumor cells provides a very complex puzzle. In some cases,

increased RNF11 levels would appear to be a beneficial adaptation while other functions of RNF11 suggest that overexpression would be harmful to a cell and lead to accelerated proliferation and cancer development. It appears that RNF11 function is likely too complex to justify a blanket statement of whether RNF11 overexpression is a favorable or unfavorable adaptation. It appears that other factors (i.e. phosphorylation) can influence normal RNF11 function. However, it may be that the upregulation of RNF11 represents a mechanism whereby a cell can cope with some of the alterations in the early stages of cancer development. Much work is necessary to establish the relevance of RNF11 in either preventing or promoting disease progression. However, it does appear that in certain epithelial cancers, RNF11 can play an important role in determining disease severity.

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## Calcium-regulated changes in mitochondrial phenotype in skeletal muscle cells

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**Freyssenet, Damien, Isabella Irrcher, Michael K. Connor, Martino Di Carlo, and David A. Hood.** Calcium-regulated changes in mitochondrial phenotype in skeletal muscle cells. *Am J Physiol Cell Physiol* 286: C1053–C1061, 2004. First published January 7, 2004; 10.1152/ajpcell.00418.2003.—Cytochrome *c* expression and mitochondrial biogenesis can be invoked by elevated intracellular  $\text{Ca}^{2+}$  in muscle cells. To characterize the potential role of  $\text{Ca}^{2+}$  as a messenger involved in mitochondrial biogenesis in muscle, we determined the effects of the  $\text{Ca}^{2+}$  ionophore A-23187 on the expression of nuclear- and mitochondrially encoded genes. Treatment of myotubes with 1  $\mu\text{M}$  A-23187 for 48–96 h increased nuclear-encoded  $\beta$ -subunit  $\text{F}_1\text{ATPase}$  and malate dehydrogenase (MDH) mRNA levels by 50–100% ( $P < 0.05$ ) but decreased mRNA levels of glutamate dehydrogenase (GDH) by 19% ( $P < 0.05$ ). mRNA levels of the cytochrome *c* oxidase (COX) nuclear-encoded subunits IV, Vb, and VIc were unchanged, whereas the mitochondrially encoded subunits COX II and COX III were decreased by 30 and 70%, respectively ( $P < 0.05$ ). This was paralleled by a 20% decrease ( $P < 0.05$ ) in COX activity. These data suggest that cytoplasmic  $\text{Ca}^{2+}$  differentially regulates the mRNA level of nuclear and mitochondrial genes. The decline in COX II and III mRNA may be mediated by Tfam, because A-23187 modestly reduced Tfam levels by 48 h. A-23187 induced time-dependent increases in Egr-1 mRNA, along with the activation of ERK1/2 and AMP-activated protein kinase. MEK inhibition with PD-98059 attenuated the increase in Egr-1 mRNA. A-23187 also increased Egr-1, serum response factor, and Sp1 protein expression, transcription factors implicated in mitochondrial biogenesis. Egr-1 overexpression increased nuclear-encoded cytochrome *c* transcriptional activation by 1.5-fold ( $P < 0.05$ ) and reduced GDH mRNA by 37% ( $P < 0.05$ ) but had no effect on MDH or  $\beta$ -subunit  $\text{F}_1\text{ATPase}$  mRNA. These results indicate that changes in intracellular  $\text{Ca}^{2+}$  can modify mitochondrial phenotype, in part via the involvement of Egr-1.

mitochondrial biogenesis; malate dehydrogenase; cytochrome *c* oxidase mitochondrial transcription factor-A; early growth response gene-1; glutamate dehydrogenase

THE MAMMALIAN MITOCHONDRION contains its own genome encoding two ribosomal (r)RNAs, 22 transfer RNAs, and 13 mRNAs that participate in the formation of important components of the mitochondrial respiratory chain (5). Other mitochondrial proteins are derived from the nuclear genome, translated in the cytosol, and subsequently imported into the mitochondria. The dual genomic organization required for the synthesis and assembly of nascent and functional multisubunit holoenzyme complexes within mitochondria suggests the involvement of a coordinated mechanism. Earlier studies (23, 41)

have shown that an increase in the expression of nuclear- and mitochondrial-encoded mRNAs occurs in rat skeletal muscle in response to chronic electrical stimulation. It has also been shown that these increases can be dissociated to some degree by the manipulation of thyroid status (21, 40), depletion of mitochondrial (mt)DNA (3, 28, 33), or by the inhibition of mitochondrial translation (9).

We have reported that cytochrome *c* gene expression was induced in skeletal muscle cells in response to treatment with the  $\text{Ca}^{2+}$  ionophore A-23187, providing the potential identification of a contractile activity-induced intracellular signal, which could induce mitochondrial biogenesis in skeletal muscle (15). Subsequently, it has been shown that the intermittent exposure of muscle cells to the  $\text{Ca}^{2+}$  ionophore ionomycin or to caffeine (34, 35), which stimulates the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum, induces the expression of genes involved in mitochondrial biogenesis. The orchestration of mitochondrial biogenesis in muscle via the  $\text{Ca}^{2+}$ -induced activation of intracellular signals has also been shown in transgenic mice selectively expressing a constitutively active form of  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase IV in muscle (42). Thus changes in intracellular  $\text{Ca}^{2+}$  have emerged as important signals for the synthesis of nuclear proteins leading to mitochondrial biogenesis. However, for  $\text{Ca}^{2+}$  to be viewed as a dominant signal for the synthesis of mitochondria in muscle, the generalizability of this response to multiple nuclear genes encoding mitochondrial proteins, as well to their potential regulating transcription factors, should be evident. Thus in this study, we extended our analysis of the involvement of calcium-mediated regulation of gene expression in skeletal muscle mitochondrial biogenesis by determining the effect of A-23187 on the expression of the number of nuclear genes encoding mitochondrial proteins, including important nuclear-encoded transcription factors, such as the early growth response gene-1 (Egr-1), specificity protein-1 (Sp1), serum response factor (SRF), and mitochondrial transcription factor A (Tfam), in addition to the expression of mitochondrial-encoded cytochrome *c* oxidase (COX) subunits II and III. Because a marked enhancement of Egr-1 mRNA was noted in response to A-23187, we subsequently examined the effect of Egr-1 overexpression on the expression of nuclear genes encoding mitochondrial proteins. Finally, because the activation of AMP-activated protein kinase (AMPK) has also been shown to increase mitochondrial biogenesis (43), we investigated the possibility of an interaction between  $\text{Ca}^{2+}$  levels and AMPK activity.

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## MATERIALS AND METHODS

**Cell culture.** L<sub>6</sub>E<sub>9</sub> cells were cultured on 100 mm gelatin-coated tissue culture plates containing DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO<sub>2</sub> in air. All cell culture materials were purchased from Sigma (St. Louis, MO). At ~60% confluence, cells were switched to differentiation medium (DMEM supplemented with 5% heat-inactivated horse serum and 1% penicillin/streptomycin). Alternatively, cells were transfected (see below) before differentiation. All treatments were carried out when myotubes reached 80–90% confluence (15).

**Drugs and treatments.** All experiments were done with differentiated cells (myotubes) and were matched with vehicle-treated control myotubes. A-23187 and ruthenium red were purchased from Sigma. They were prepared as stock solutions of 0.25 mM in Me<sub>2</sub>SO (Sigma) and 7 mM in distilled water, respectively. PD-98059 was obtained from CalBiochem (San Diego, CA) and prepared as 25 mM solution in Me<sub>2</sub>SO. For most experiments, A-23187 was used at a concentration of 1  $\mu$ M for 48 h. The dose-dependent effect of A-23187 on malate dehydrogenase mRNA level was evaluated with concentrations ranging from 0.25 to 1.50  $\mu$ M. The effect of time was evaluated by incubating myotubes with 1  $\mu$ M A-23187 for 8, 24, 48, 72, or 96 h. The effect of ruthenium red was evaluated by preincubating myotubes for 24 h with 10  $\mu$ M ruthenium red before the treatment with 1  $\mu$ M A-23187 was started for 72 h. The effect of the MEK inhibitor was evaluated by preincubating myotubes for 30 min with 10  $\mu$ M PD-98059 before starting the treatment with 1  $\mu$ M A-23187 for 2, 4, and 8 h.

**RNA isolation, Northern blotting, and radiolabeled probes.** Total RNA was extracted as previously described (15). The concentration and purity were determined by ultraviolet spectrophotometry at 260 and 280 nm, respectively. Total RNA (10  $\mu$ g) was separated on denaturing formaldehyde-1% agarose gel and transferred overnight to nylon membranes (Hybond N, Amersham; Arlington Heights, IL) (10). Radiolabeled probes were generated by random primer labeling in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham-Pharmacia Biotech, Mississauga, Ontario, Canada), as done previously (15). Membranes were prehybridized overnight at 42°C. Blots were then incubated overnight at 42°C after the addition of the appropriate radiolabeled probe (2  $\times$  10<sup>6</sup> cpm) encoding COX subunits II, III, IV, Vb, VIc,  $\alpha$ -actin,  $\beta$ -subunit of F<sub>1</sub>ATPase, malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), or Egr-1. The blots were then rinsed and washed 3  $\times$  10 min at room temperature with 2 $\times$  SSC (0.15 M NaCl/0.030 M sodium citrate), 0.1% SDS, followed by 1  $\times$  15 min at 55°C and 1  $\times$  15 min at 65°C. The final wash for membranes hybridized with cytochrome oxidase subunit II or the  $\beta$ -subunit of F<sub>1</sub>ATPase was 60°C for 15 min. Signals were corrected for loading using hybridization signals produced by 18S rRNA and were quantified by electronic autoradiography.

**Western blot analysis.** Total protein extracts from cultured L<sub>6</sub>E<sub>9</sub> myotubes treated were treated for varying times up to 48 h with 1  $\mu$ M A-23187 were harvested and electrophoresed through SDS-polyacrylamide gels, and electroblotted onto nitrocellulose membranes (Amersham, Baie D'Urfé, Québec, Canada). Overnight incubations with antibodies diluted in blocking buffer directed toward phospho-AMPK- $\alpha$  (1:400; New England Biolabs, Mississauga, Ontario, Canada) or total AMPK- $\alpha$  (1:750; New England Biolabs), cytochrome *c* (1:750) (34), Egr-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-extracellular regulated kinases-1 and -2 (ERK1 and ERK2; 1:400; New England Biolabs) or total ERK1/2 (1:1,000; New England Biolabs), Sp1 (1:500; Santa Cruz Biotechnology), SRF (1:1,000; Santa Cruz Biotechnology), and Tfam (1:1,000) (15) were followed by incubations at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase, visualized with enhanced chemiluminescence and quantified using SigmaGel (Jandel, San Rafael, CA).

**Cell transfection.** L<sub>6</sub>E<sub>9</sub> myoblasts were transfected with either the -66 or the -726 bp cytochrome *c* promoter/luciferase construct (2.5  $\mu$ g/100 mm dish) when they reached 70% confluence. Cells were cotransfected with p-cytomegalovirus/Renilla luciferase (5 ng/dish) to correct for differences in transfection efficiency. Where applicable, wild-type Egr-1, or empty vector expression vectors were transfected (2.5  $\mu$ g/dish) in combination with cytochrome *c* promoter/luciferase constructs. The wild-type Egr-1 expression vector contains the full-length Egr-1 cDNA under the transcriptional control of the cytomegalovirus promoter. The mutant construct is expressed at similar levels but lacks DNA binding activity and has been previously described (15). The total amount of DNA added was maintained constant in all transfected cells. Transfections were done with the use of the poly-L-ornithine method, followed by a Me<sub>2</sub>SO shock (14) or with the use of Lipofectamine 2000 (Invitrogen, Mississauga, Ontario) following the manufacturer's recommendations. Cells were then differentiated by switching to a low-serum medium and subsequently scraped for either total RNA isolation or for measurement of luciferase activities. Firefly and renilla luciferase activities were measured using luminometer (Lumat 9507, Berthold) and a Promega (Madison, WI) Dual Luciferase Assay kit, according to the manufacturer's instructions.

**Cell extracts and enzyme activities.** Cells were harvested after 72 h of treatment and centrifuged at 4°C for 5 min. The cell pellet was then resuspended with 100  $\mu$ l of 0.1 M KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>PO<sub>4</sub> buffer (pH 7.2) containing 2 mM EDTA. The cells were then sonicated (4  $\times$  10 s) on ice and centrifuged at 4°C for 5 min. Protein concentrations of the resulting supernates were obtained by determining absorbance at 280 nm. COX enzyme activity was measured by the rate of oxidation of fully reduced cytochrome *c* (22). MDH activity was measured as the rate of reduction of NAD (20).

**ATP measurements.** Control and 48-h-treated L<sub>6</sub>E<sub>9</sub> myotubes were trypsinized and centrifuged for 4 min at 2,000 g. The pellet was deproteinized with 6 M perchloric acid, 80  $\mu$ l of cold PBS, and centrifuged for 5 min at 14,000 g. The supernatant was neutralized with 2 M KOH and centrifuged for 5 min at 14,000 g. The resulting supernatant fraction was used to determine ATP production using a Berthold Luminometer. All measurements were normalized to total protein content.

**Statistical analysis.** Values were expressed as means  $\pm$  SE. One-way analyses of variance, followed by Scheffé's post hoc test ( $P < 0.05$ ), were used for multiple data comparisons (Figs. 1, 2A, and 4B). Student's *t*-test was used for comparing two groups of data (slopes in Fig. 2A, COX II mRNA data), as appropriate.

## RESULTS

**Effect of A-23187 treatment on MDH expression in L<sub>6</sub>E<sub>9</sub> cells.** We initially characterized the response of the nuclear-encoded mitochondrial matrix enzyme MDH to A-23187 treatment, similar to our previous work with cytochrome *c* (15). The treatment of myotubes with the Ca<sup>2+</sup> ionophore A-23187 for 48 h increased MDH mRNA level in a time-dependent fashion, such that an ~50–100% increase ( $P < 0.05$ ) was evident between 48–96 h of treatment (Fig. 1A). This effect was also reflected at the protein level as MDH enzyme activity was significantly increased by 80% ( $P < 0.05$ ;  $n = 4$ ) in response to A-23187 treatment for 48 h (Fig. 1A). The effect of A-23187 on MDH mRNA expression was also concentration-dependent up to 1.5  $\mu$ M and was abolished when myotubes were preincubated with the extracellular chelating agent EGTA (Fig. 1B). This strongly suggests that the effect of A-23187 was mediated through Ca<sup>2+</sup> mobilization from the extracellular pool.

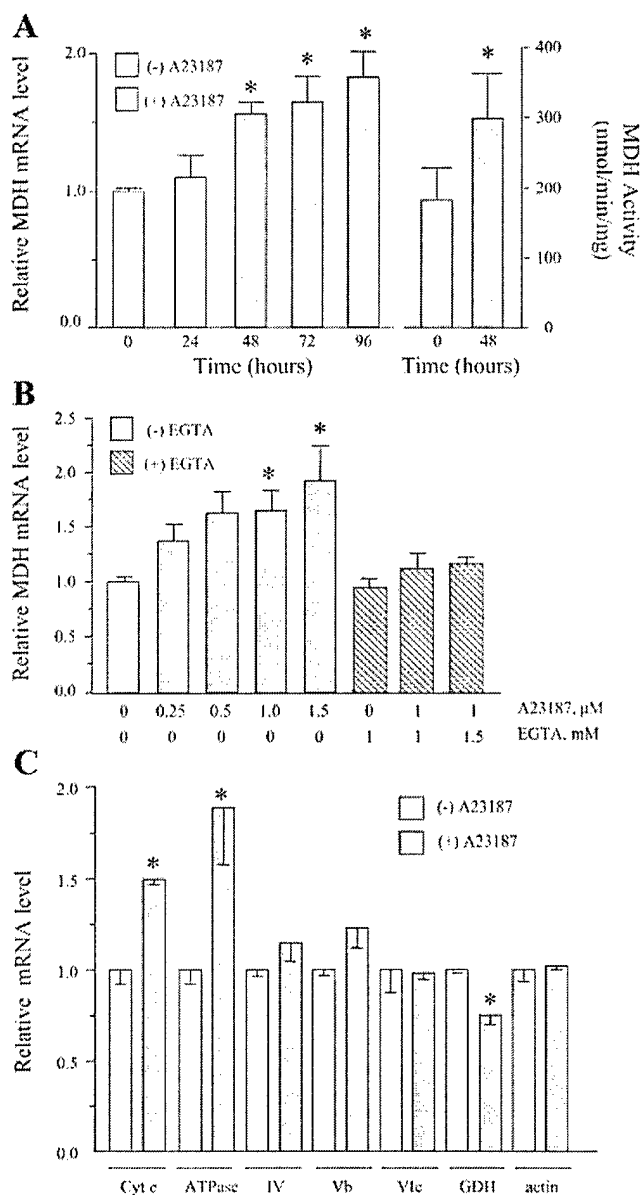


Fig. 1. Effect of A-23187 on the mRNA levels of nuclear genes encoding mitochondrial proteins. *A*: quantification of Northern blot analyses of malate dehydrogenase (MDH) mRNA levels and MDH enzyme activities as a function of time post-A-23187 treatment (1 μM; \* $P$  < 0.05 vs. control), and (*B*) as a function of A-23187 concentration, incubated for 48 h (\* $P$  < 0.05 vs. 0 μM A-23187). The effect of preincubation (5 h) with EGTA is also shown. *C*: effect of 1 μM A-23187 (48 h) on mRNA levels encoding cytochrome *c* (Cyt *c*; Ref. 15), β-F<sub>1</sub>ATPase, cytochrome *c* oxidase (COX) IV, COX Vb, COX Vlc, glutamate dehydrogenase (GDH), and α-actin [\* $P$  < 0.05 vs. (-) A-23187;  $n$  = 3–5 experiments/condition].

*A-23187 differentially regulates nuclear genes encoding mitochondrial proteins.* A-23187 treatment of myotubes for 48 h elicited a similar response when Northern blot analyses were performed with a probe encoding the β-subunit of the F<sub>1</sub>ATPase (Fig. 1C). Quantification of this effect revealed a 90% increase ( $P$  < 0.05) in mRNA expression level. In contrast to the response observed for cytochrome *c* (15), MDH and the β-subunit of F<sub>1</sub>ATPase, no changes in the COX IV,

COX Vb, and COX Vlc mRNA levels were observed in response to 48 h of A-23187 treatment (Fig. 1C), whereas glutamate dehydrogenase (GDH) mRNA expression was reduced by 19% ( $P$  < 0.05). In addition, α-actin mRNA, used to assess the specificity of the effect of A-23187 on nuclear genes encoding mitochondrial proteins, was expressed at similar levels both in control and A-23187-treated cells (Fig. 1C).

*Effect of A-23187 on mitochondrially encoded COX II and III mRNA expression, and COX enzyme activity.* The lack of response among nuclear-encoded COX subunit mRNA expression to A-23187 prompted us to investigate the expression of the mitochondrially encoded COX subunits II and III. Surprisingly, COX II mRNA expression declined by ~30% ( $P$  < 0.05), whereas COX III mRNA level was more markedly affected, exhibiting an ~70% decrease ( $P$  < 0.05) when myotubes were incubated with 1 μM A-23187 for 48 h (Fig. 2A). This effect does not appear to depend on Ca<sup>2+</sup> entry into the organelle via the mitochondrial Ca<sup>2+</sup> uniporter, the major route of Ca<sup>2+</sup> entry into the organelle (18, 19, 29) because preincubation of myotubes for 24 h with 10 μM ruthenium red

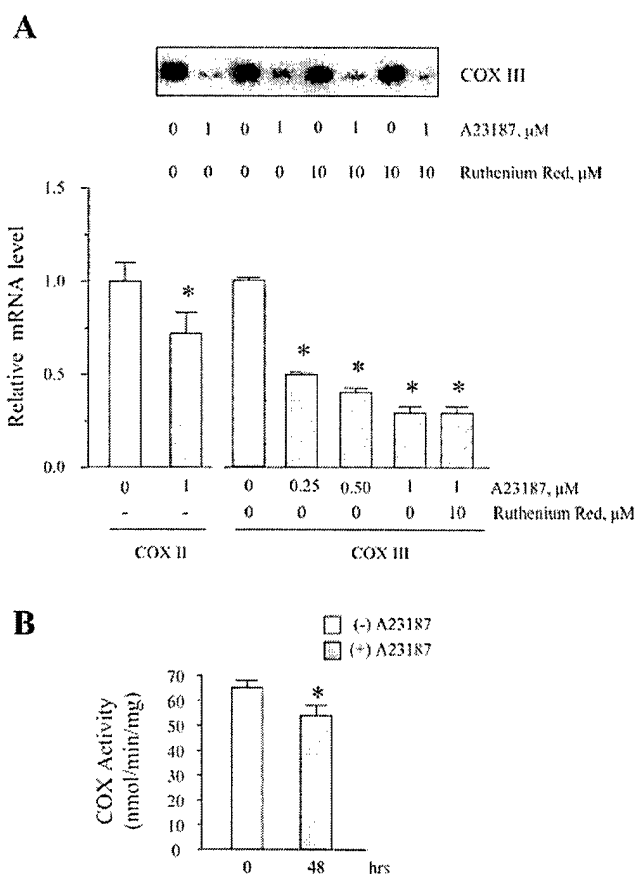


Fig. 2. Effect of A-23187 on the expression of mitochondrially encoded COX subunits. *A*: representative Northern blot showing the effect of A-23187 on COX II and III mRNA expression after 48 h of A-23187 (or vehicle) treatment. COX III mRNA expression was also evaluated in a subset of A-23187-treated cells pretreated with ruthenium red (10 μM). The effects of A-23187 and ruthenium red were quantified and graphically represented (\* $P$  < 0.05 vs. 0 μM A-23187;  $n$  = 3 to 4 experiments/condition). *B*: effect of A-23187 on COX activity in cells treated for 48 h with 1 μM A-23187 (\* $P$  < 0.05 vs. 0 h;  $n$  = 4 experiments).

did not change the response elicited by 48 h of A-23187 treatment on COX III mRNA (Fig. 2A). Furthermore, these data also suggest that the A-23187-mediated effect is initiated outside the mitochondrion, possibly via changes in the expression of nuclear-encoded transcription factors (see below). The relevance of the decrease in COX II and III mRNA expression was also reflected at the protein level, because A-23187 induced a 20% decrease in COX activity ( $n = 4$ ;  $P < 0.05$ ; Fig. 2B). Taken together, these data suggest that an increase in intracellular cytosolic Ca<sup>2+</sup> concentration mediated by A-23187 differentially regulates the expression of genes encoding mitochondrial proteins.

**Effect of A-23187 on Egr-1 expression.** We investigated the effect of A-23187 on the mRNA level of Egr-1, a transcription factor that has putative binding sites within the upstream promoter regions of several nuclear genes encoding mitochondrial proteins (32), including GDH (12). A sequence similar to, but not identical to the consensus site, is also found in the cytochrome *c* promoter. A rapid 100–150% increase ( $P < 0.05$ ,  $n = 3$  experiments) in Egr-1 mRNA was observed as early as 1 h posttreatment, was maintained at 2 h, and declined by 4 h (Fig. 3A). This early response is consistent with the induction of Egr-1 as an upstream event in a cascade of reactions leading to changes in gene expression in response to A-23187. Interestingly, the increase in Egr-1 mRNA expression by 2 h was abolished by preincubation of the cells for 30 min with 12.5  $\mu$ M PD-98059, a MEK inhibitor (Fig. 3A). At 4 h, when the effect of A-23187 was reduced to ~30% ( $n = 3$ ), the effect of MEK inhibition was more pronounced. This finding is consistent with other results, in which MEK inhibition with PD-98059 led to a dose-dependent reduction in baseline Egr-1 mRNA levels (Lowe S and Hood DA, unpublished observations). We then evaluated whether the downstream targets of MEK, the extracellular regulated kinases-1 and 2 (ERK1/2), could be activated via A-23187. ERK 2 activation was evident by 2 h, was significantly increased ~2.5-fold ( $P < 0.05$ ) by 4 h, and returned to control levels by 8 h of treatment. The kinetics of these responses, along with the inhibition data, suggest that the induction of Egr-1 mRNA levels by A-23187 is mediated by both ERK1/2-dependent and -independent effects.

To investigate whether the early A-23187-mediated increase in Egr-1 mRNA could lead to subsequent changes in Egr-1 protein expression, we examined Egr-1 protein expression following treatment with A-23187 for 8 and 48 h. A-23187 treatment for 8 h increased Egr-1 protein 3.5-fold ( $P < 0.05$ ), a response that was diminished by 48 h (Fig. 4, A and B).

**Time-dependent changes in Sp1, SRF, and Tfam protein expression after A-23187 treatment.** We investigated the possibility that the effect of A-23187 on COX II and III mRNA levels (Fig. 2A) was mediated via Ca<sup>2+</sup>-induced changes in the levels of Tfam, a nuclear-encoded transcription factor protein that is imported into mitochondria and binds to the light and heavy-strand promoter of mtDNA (36). We therefore evaluated Tfam protein expression after 8 and 48 h of exposure to A-23187. No change in Tfam protein was observed after 8 h of treatment. This is consistent with our observations of a lack of change in Tfam mRNA expression for up to 12 h of treatment with A-23187 (data not shown). However, a 32% decrease ( $P < 0.05$ ) by 48 h was observed, in parallel with the overall decrease in the mitochondrially encoded COX II and III sub-

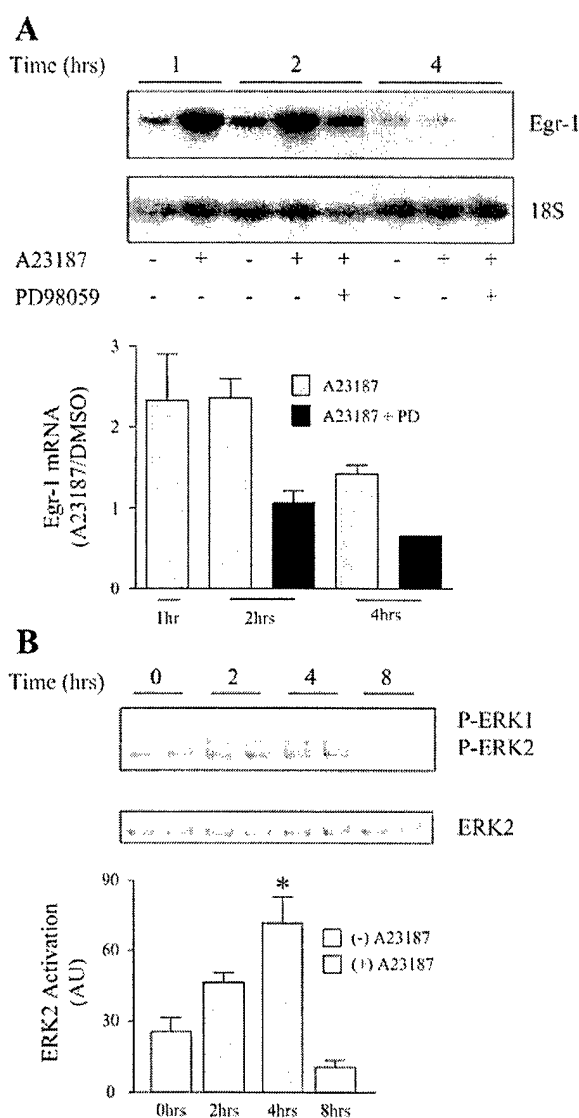


Fig. 3. Effect of A-23187 on early growth-response gene-1 (Egr-1) mRNA expression and mitogen-activated protein (MAP) kinase activation. **A:** Egr-1 mRNA expression as a function of time posttreatment with A-23187 (+) or vehicle (-). Preincubation (30 min) with the MEK inhibitor PD-98059 (PD; 10  $\mu$ M) is also shown and is representative of three experiments. 18S rRNA is also shown to verify gel loading. **B:** representative Western blot (75  $\mu$ g/lane) showing the effect of A-23187 on ERK 1 and 2 MAP kinase activation, and total ERK2 protein levels as a function of time posttreatment. ERK2 kinase activation was quantified, and a summary of repeated experiments ( $n = 3$ ) is shown. \* $P < 0.05$  vs. 0 h.

units, and the overall decrease in COX activity observed at that time.

We extended our investigation beyond Egr-1 and Tfam, to identify additional calcium-regulated transcription factors that may be responsible for mediating changes in both nuclear and mitochondrial gene expression. We chose to investigate Sp1 because almost all nuclear genes encoding mitochondrial proteins, including Tfam and cytochrome *c*, contain Sp1 binding sites within their promoter regions (7, 10, 14, 20, 32, 39). We also investigated the effect of A-23187 on serum response factor (SRF) protein expression, as it has been implicated in

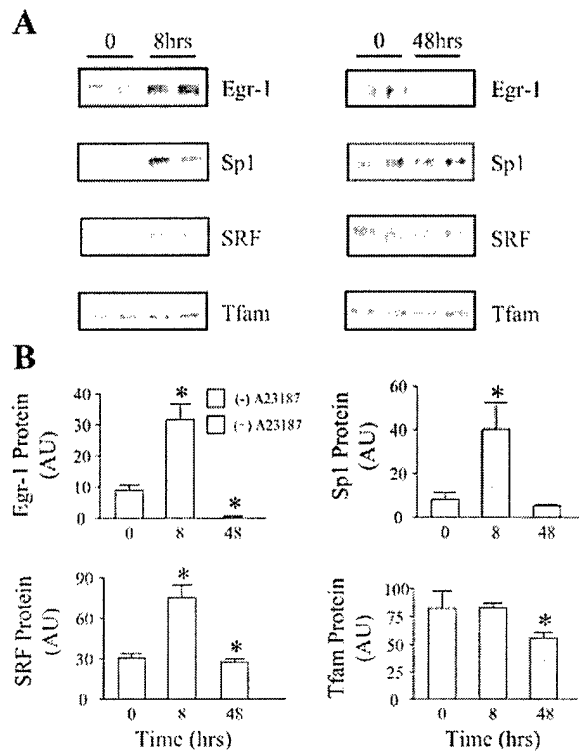


Fig. 4. Effect of A-23187 treatment on transcription factor protein expression. *A*: representative Western blots (50–100  $\mu$ g/lane) showing the effect of A-23187 on Egr-1, specificity protein-1 (Sp1), serum response factor (SRF), and mitochondrial transcription factor A (Tfam) after 8 and 48 h of treatment. Data obtained at the two time points were collected in separate experiments each compared with appropriate controls (time 0), resulting in variable signal intensities when compared between 8 and 48 h. *B*: effect of 8 and 48 h of A-23187 treatment was quantified, and a summary of repeated experiments ( $n = 3$ ) is shown. \* $P < 0.05$  vs. 0 h.

regulating the transcription of Egr-1 (8), and it has a binding site within the F<sub>1</sub>ATPase promoter (32). A-23187 treatment for 8 h increased ( $P < 0.05$ ) Sp1 and SRF protein levels by 4.9- and 2.4-fold, respectively (Fig. 4, *A* and *B*). The effect of A-23187 on SRF and Sp1 expression was completely dissipated by 48 h (Fig. 4, *A* and *B*).

**Effect of Egr-1 overexpression on mRNA levels, cytochrome *c* transcriptional activation, and protein expression.** We hypothesized that the A-23187-mediated changes in Egr-1 mRNA and protein expression could lead to an increase in the expression of nuclear genes encoding mitochondrial proteins. To evaluate this, we overexpressed Egr-1 and measured MDH,  $\beta$ -F<sub>1</sub>ATPase, and GDH mRNA levels. Although no changes in the levels of MDH or  $\beta$ -F<sub>1</sub>ATPase mRNA were observed (Fig. 5*A*), Egr-1 overexpression decreased GDH mRNA expression by 37% ( $P < 0.05$ ; Fig. 5, *A* and *B*).

Because the cytochrome *c* promoter contains potential binding sites for Egr-1 based on sequence similarities with Sp1, we also sought to determine the effect of Egr-1 overexpression on cytochrome *c* transcriptional activation. To verify this, both the minimal (–66 bp) and the full (–726 bp) length cytochrome *c* promoter/luciferase constructs were cotransfected with Egr-1 into L<sub>6</sub>E<sub>9</sub> cells. Egr-1 overexpression had no effect on cytochrome *c* transcriptional activation of the minimal promoter,

but increased the full-length cytochrome *c* promoter activity 1.5-fold over the empty vector-matched control (Fig. 5*C*).

**Effect of A-23187 treatment on AMPK- $\alpha$  activation.** The intracellular signals involved in stimulating mitochondrial biogenesis in skeletal muscle likely involve crosstalk between multiple signaling pathways. This was most recently demonstrated in mice expressing a muscle-specific dominant negative AMPK transgene, where the expression of the calcium-regulated CamKIV appeared to be dependent on AMPK- $\alpha$  (43). We therefore explored the possibility that AMPK- $\alpha$  activation may also be calcium dependent. Forty-eight hours of A-23187 treatment dramatically increased ( $P < 0.05$ ) AMPK- $\alpha$  activation by 10-fold (Fig. 6*A*). To confirm that chronic elevations in intracellular Ca<sup>2+</sup> did not compromise cellular energy status, ATP levels were measured in cells treated with A-23187 for 48 h. No significant change in ATP concentrations were observed, suggesting that A-23187 had no effect in compromising the energy status of the cells (Fig. 6*B*).

## DISCUSSION

Our recent finding that elevations in intracellular Ca<sup>2+</sup> concentration within skeletal muscle cells increased cyto-

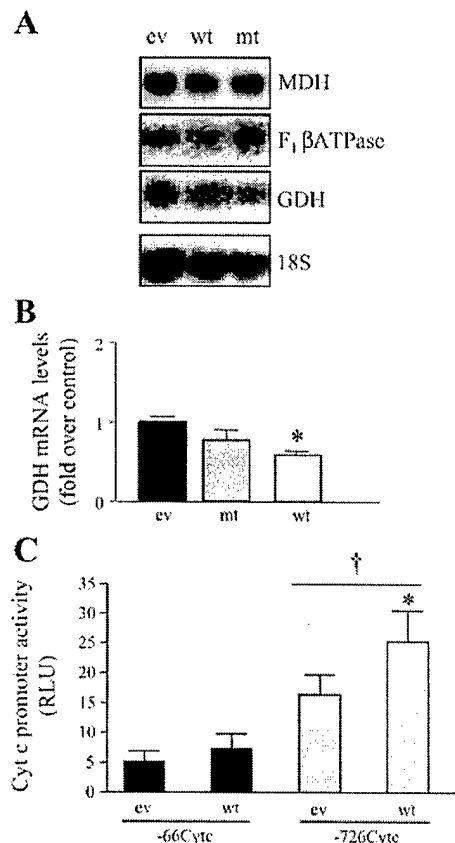


Fig. 5. Effect of Egr-1 overexpression on mRNA expression and cytochrome *c* transcriptional activation. *A*: MDH,  $\beta$ -F<sub>1</sub>ATPase, and GDH mRNA expression in the presence of wild-type (wt) or mutant (mt) Egr-1 overexpression, or with empty vector (ev). *B*: quantification of GDH mRNA obtained from multiple Northern blots as shown in *A* (\* $P < 0.05$  vs. ev;  $n = 5$  experiments). *C*: cytochrome *c* transcriptional activation of the full (–726 bp) and minimal (–66 bp) promoters in the presence of wild-type Egr-1 overexpression or empty vector. † $P < 0.05$  –726Cyt c vs. –66Cyt c; \* $P < 0.05$  wt vs. ev.

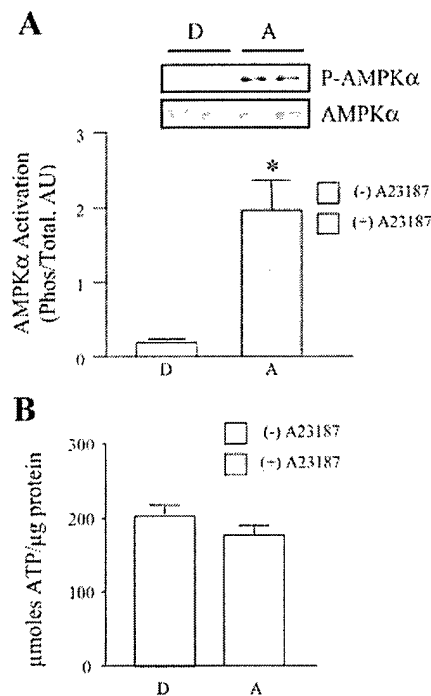


Fig. 6. *A*: representative Western blots showing the effect of 48 h of A-23187 on AMP-activated protein kinase (AMPK- $\alpha$ ) activation and total AMPK- $\alpha$  protein levels. Kinase activation, measured as a fold change of the phosphorylated protein over the total protein, was quantified and a summary of repeated experiments ( $n = 3$ ) is shown; \* $P < 0.05$  vs. DMSO. *B*: immediately after A-23187 treatment, cells were harvested and ATP levels were measured as described in MATERIALS AND METHODS. A summary of two experiments, each measurement repeated in triplicate, is shown.

chrome *c* transcriptional activation and mRNA levels (15) led us to examine whether this Ca<sup>2+</sup> effect was specific to cytochrome *c*, or common to other nuclear genes encoding mitochondrial proteins. Our results show that, similar to cytochrome *c*, the nuclear-encoded MDH and F<sub>1</sub>β-ATPase respond to elevated intracellular Ca<sup>2+</sup> levels by increasing their mRNA concentrations, whereas GDH mRNA expression decreased in response to the same treatment. We also show that the mRNAs encoding subunits of the COX holoenzyme are differentially regulated by changes in intracellular Ca<sup>2+</sup> concentrations. In particular, we show that A-23187 treatment decreases the mRNA level of mitochondrially encoded genes COX II and COX III of cytochrome oxidase, whereas the nuclear-encoded subunits IV, Vb, and VIc of COX remained unaffected. This pattern of change in mRNA level was meaningful because it was reflected at the protein level by elevated MDH and reduced COX enzyme activities. Thus our findings uniquely show that elevating intracellular Ca<sup>2+</sup> differentially regulates mRNA levels of genes encoding mitochondrial proteins and therefore suggest that Ca<sup>2+</sup> does not constitute a signal that can lead to stoichiometric increases in all mitochondrial proteins, resulting in mitochondrial biogenesis.

Although a continuously elevated cytosolic Ca<sup>2+</sup> concentration does not compare with the transient physiological oscillations in Ca<sup>2+</sup> observed in contracting muscle cells, continuously elevated levels have been observed in specific pathological states and under conditions of muscle adaptation.

For example, muscle cells that have been depleted of mtDNA, and fibroblast cells from mitochondrial encephalopathy and lactic acid syndrome patients, which harbor a mtDNA tRNA<sup>leu</sup> mutation, both exhibit chronically elevated levels of cytosolic Ca<sup>2+</sup>. These cells exhibit significant changes in mitochondrial phenotype (3, 31). Furthermore, cytosolic Ca<sup>2+</sup> levels are also markedly elevated in skeletal muscle subject to chronic electrical stimulation, and it is well known that one of the consequences of chronic stimulation is mitochondrial biogenesis (6, 37). Thus the A-23187-mediated elevation in cytosolic Ca<sup>2+</sup> concentrations, which can be sustained for up to 16 days after treatment (26), models cellular environments in which changes in mitochondrial phenotype can be produced.

In skeletal muscle cells, increases in cytosolic Ca<sup>2+</sup> concentration elicited by muscle-specific agents such as nicotinic agonists, KCl depolarization in Ca<sup>2+</sup>-free medium, or caffeine are amplified four- to sixfold within the mitochondrial matrix (4). This raises the important question as to whether Ca<sup>2+</sup> is a direct regulator of mitochondrial gene expression via actions within the organelle, on the mitochondrial transcription machinery, or whether it acts outside the mitochondrion (i.e., in the nucleus or cytosol) to modify the expression or activity of molecules that are subsequently imported to alter mitochondrial gene expression. Under physiological conditions, mitochondrial Ca<sup>2+</sup> influx is mediated by a uniporter that facilitates the diffusion of Ca<sup>2+</sup> down its electrochemical gradient (18, 19, 29). Ruthenium red, a potent noncompetitive inhibitor of the Ca<sup>2+</sup> uniporter, was used here to determine whether Ca<sup>2+</sup> entry via this route could account for the effect of A-23187 on COX II and III mRNA levels. Our results demonstrate that 10 μM ruthenium red did not abolish the effect mediated by A-23187 on COX III mRNA levels. Because Ca<sup>2+</sup> influx into mitochondria via the uniporter can be attenuated and completely blocked at concentrations much less than the dose used in this study (19), we conclude that Ca<sup>2+</sup> entry via this pathway is not responsible for the effect observed, and that this effect likely originates external to the organelle.

The nuclear-encoded transcription factor Tfam is an important protein involved in the transcription of the mitochondrial genome (36). In the present study, Tfam mRNA and protein levels were determined to assess whether changes in Tfam expression could explain the decrease in mitochondrial-encoded COX II and III mRNA levels. Although early time points revealed no change in Tfam mRNA or protein levels, Tfam protein expression was reduced following a longer treatment period. This decrease coincided precisely with decreases in COX II and III mRNA expression, as well as with the decrease in COX activity reported here. Thus these data support our hypothesis that the A-23187-mediated effect on mitochondrial gene transcription originates outside the mitochondrion via a decrease in Tfam protein expression. The use of a number of different experimental conditions has shown that a positive relationship exists between Tfam protein expression and the regulation of mtDNA-derived transcripts of the COX holoenzyme. For example, chronic stimulation of skeletal muscle *in vivo* has been shown to increase Tfam protein expression. This corresponded to an increase in Tfam import into mitochondria, an increase in Tfam/DNA binding to the D-loop of mtDNA, and corresponding increases in mitochondrial-encoded COXIII mRNA expression (17). In addition, elevated Tfam expression in cancer cells was matched by parallel

increases in mitochondrial-encoded COXI and COXII mRNA expression (13). Conversely, when Tfam expression was experimentally decreased (24), the expression of mitochondrially encoded COX subunits was also reduced. Our findings in the present study using A-23187 to influence changes in gene expression also support this relationship.

Egr-1 is an immediate-early gene, which has been previously shown to increase in response to contractile activity (2, 10, 30) and also to increased intracellular Ca<sup>2+</sup> (1). Importantly, putative binding sites for Egr-1 in the upstream region of GDH (12) and possibly cytochrome *c* exist, suggesting that Egr-1 may be involved in the Ca<sup>2+</sup>-mediated signal leading to mitochondrial biogenesis. In the present study, the response of Egr-1 mRNA and protein expression to elevated internal Ca<sup>2+</sup> was large and rapid, and it occurred before any changes in the mRNA for cytochrome *c* (15), MDH, GDH, or F<sub>1</sub>β-ATPase, consistent with our hypothesis. Thus, to investigate a direct role for Egr-1, we evaluated the effect of Egr-1 overexpression on cytochrome *c* transcriptional activation and the mRNA expression of GDH, examples of nuclear-encoded gene products, which increase and decrease, respectively, in response to A-23187. Here we show that Egr-1 overexpression transcriptionally activated the full-length cytochrome *c* promoter and also led to a marked decrease in GDH mRNA levels. These data support a role for this transcription factor in the regulation of nuclear genes encoding mitochondrial proteins, leading to an altered organelle phenotype. Although putative Egr-1 binding sites within the cytochrome *c* promoter are thought to exist, multiple Sp1 sites have been identified in the cytochrome *c* promoter, and it is well known that Egr-1 and Sp1 can compete for similar GC-rich promoter elements (25). Thus, increases in Egr-1, via initial elevations in intracellular Ca<sup>2+</sup>, represent a possible mechanism by which cytochrome *c* is transcriptionally activated. It is also known that the GDH promoter contains overlapping Egr-1 and Sp1 sites. Under these circumstances, an increase in Egr-1 expression can facilitate the displacement of Sp1 from its binding site, resulting in a reduction in mRNA expression (24), thus demonstrating that Egr-1 can act as a transcriptional repressor. The decrease in GDH mRNA expression via A-23187, and also by Egr-1 overexpression, is supportive of a Ca<sup>2+</sup>-initiated, Egr-1-mediated pathway (Fig. 7).

To establish whether additional Ca<sup>2+</sup>-regulated factors may be involved in regulating the expression of genes encoding mitochondrial proteins, we also evaluated the effect of A-23187 on Sp1 and SRF in addition to Egr-1 and Tfam because putative binding sites for these factors exist within the promoters of many nuclear genes encoding mitochondrial proteins, including COX enzyme subunits (27, 32) and Tfam (7). On the basis of Western blot analyses (Fig. 4), our results suggest that the early increase in SRF and Sp1 protein, in addition to Egr-1, may serve as additional factors that aid in the regulation of mitochondrial gene expression (Fig. 7).

The transduction of intracellular signals into changes in gene expression often occurs via the activation of signaling kinases. Several Ca<sup>2+</sup>-regulated signaling pathways have been identified, including the MAP kinases. With respect to Egr-1, we have shown that the increase in Egr-1 mRNA can be markedly attenuated in the presence of the MEK inhibitor PD-98059. Our previous work (15) has demonstrated that the cytochrome *c* transactivation mediated by A-23187 was reduced by ~40%

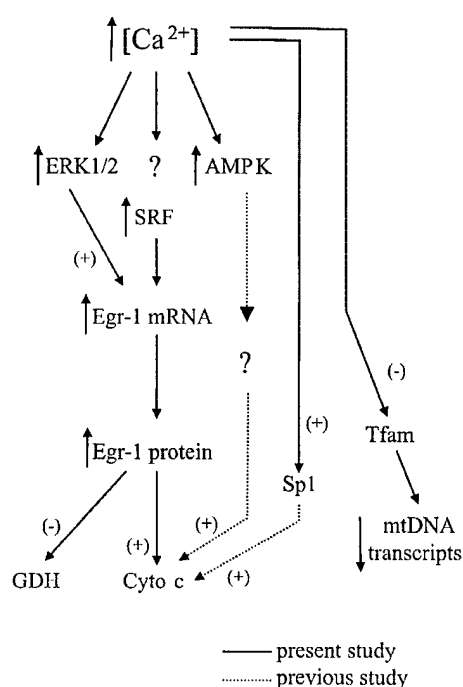


Fig. 7. Summary of Ca<sup>2+</sup>-regulated changes in gene expression leading to alterations in mitochondrial phenotype in skeletal muscle cells. A-23187 activates both MAP kinase and AMPK and increases SRF expression, leading to increases in Egr-1 mRNA and protein (7, and present study). Egr-1 upregulates cytochrome *c* transcription and downregulates GDH mRNA expression. The A-23187-induced increase in Sp1 can also affect cytochrome *c* levels (14), whereas the decrease in Tfam will result in a reduction of mitochondrial (mt)DNA transcript levels. These effects lead to an alteration in muscle mitochondrial phenotype.

in the presence of PD-98059 and that MAP kinases (ERK1 and ERK2) were rapidly and transiently activated by A-23187 (Fig. 3B). The fact that the increase in Egr-1 mRNA level was also blunted by the MEK inhibitor is consistent with the involvement of MAP kinase as an early signaling event in the activation of Egr-1 and, subsequently, cytochrome *c* expression (Fig. 7).

Finally, an interesting result of the current study is the A-23187-mediated activation of AMPK. Only one investigation to date has documented a link between Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV and AMPK (43). However, in that study it was reported that the regulation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV expression occurs downstream of AMPK activation. Our results suggest that AMPK activation occurs directly as a result of Ca<sup>2+</sup> signaling, independent of changes in cellular energy status as reflected by ATP concentration, since decreases in ATP levels, and presumably AMP levels in A-23187-treated cells were not observed. More studies are warranted to investigate the direct or indirect role of Ca<sup>2+</sup>-evoked changes in AMPK activation, and other proteins involved in mediating mitochondrial biogenesis.

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## **AKT-mediated regulation of Ring Finger Protein 11 (RNF11).**

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Ring-finger proteins serve many vital functions within the cell. We have identified RNF11, a novel 154 amino acid ring-finger containing protein, which is elevated in breast cancer. It is unclear as to whether RNF11 is regulated transcriptionally or translationally. RNF11 mRNA is low in G0 and it becomes more abundant as cells progress into G1. Within its ring-finger domain, RNF11 contains an AKT phosphorylation site (T135) that is situated within a 14-3-3 binding domain. In WM239 cells with constitutively active AKT, resulting from a PTEN deletion, RNF11 exhibits 7 distinct phosphopeptides as measured using 2-D phosphopeptide mapping. Upon treatment with the PI3-kinase inhibitor LY2940002, the phosphorylation at 1 of these sites is virtually eliminated. When T135 is mutated, phosphorylation at this same site is also eliminated, suggesting that AKT may phosphorylate RNF11 at T135. Moreover, RNF11 is recognized as a phospho-AKT substrate by immunoblotting. The 14-3-3 binding domain located within the ring finger of RNF11 may serve as an important site for the regulation of RNF11 function. RNF11 demonstrates enhanced binding to 14-3-3 in WM239 cells when compared to that seen in the parental WM35 cells. Furthermore, treatment of WM239 cells with LY294002 reduces RNF11/14-3-3 interactions. These data suggest that RNF11/14-3-3 binding is regulated by AKT and these observations are further corroborated by the increased binding of RNF11 to 14-3-3 with cotransfection of RNF11 and a constitutively active AKT into MCF-7 cells than when RNF11 is transfected alone. In addition, there is a significant reduction in 14-3-3 binding to the T135 mutant RNF11. Interestingly, cotransfection of AKT and RNF11 results in decreased expression of RNF11, which suggests a role for AKT in RNF11 degradation. In MCF-7 cells, RNF11 is predominantly localized in the cytoplasm with some nuclear RNF11. When cotransfected with constitutively active AKT, RNF11 is entirely nuclear. This suggests that AKT phosphorylation of RNF11 causes its nuclear localization or that AKT phosphorylation induces degradation of the cytoplasmic pool of RNF11. Although the precise mechanisms are not fully understood, it is clear that RNF11 function, localization and potentially degradation are regulated by AKT. This may be important in breast cancer, where active AKT is associated with poor prognosis. Disregulation of proper RNF11 function by AKT may prove to be detrimental to patient outcomes making RNF11 a potential target for novel cancer therapeutics.